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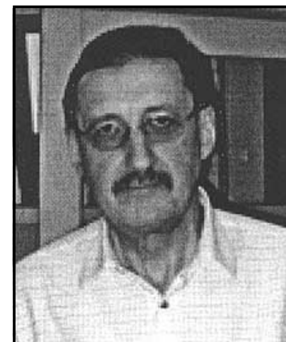
Brain Nitric Oxide and Its Dual Role in Neurodegeneration/Neuroprotection: Understanding Molecular Mechanisms to Devise Drug Approaches

Antonio Contestabile*, Barbara Monti, Andrea Contestabile and Elisabetta Ciani

Department of Biology and Department of Human and General Physiology, University of Bologna, Italy



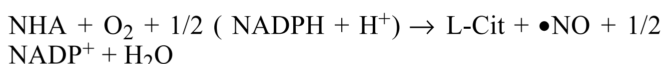
Abstract: Nitric oxide (NO) has been established as an important messenger molecule in various steps of brain physiology, from development to synaptic plasticity, learning and memory. However, NO has also been viewed as a major agent of neuropathology when, escaping controlled production it may directly or indirectly promote oxidative and nitrosative stress. The exact borderline between physiological, and therefore neuroprotective, and pathological, and therefore neurodegenerative, actions of NO is a matter of controversy among researchers in the field. This is reflected in the present status of drug research, that is focused on finding ways to block NO production, and therefore limit neuropathology, as well as on finding ways to increase NO availability and therefore elicit neuroprotection. As an unavoidable consequence, both classes of drugs are reported to have neurodegenerative or neuroprotective effects, depending on the models in which they are tested. Aim of the present paper is to provide the reader with a survey, as much complete as possible, on the main aspects of NO biology, from biochemistry and chemical reactivity to the molecular signals elicited in neural cells target of its neurodegenerative or neuroprotective action. In doing that, many controversial aspects related to basic biology and to neuropathology of NO are taken into account. In the final sections, main classes of drugs able to interfere with NO physiopathology are examined, in order to try to devise possible directions for future NO-based therapeutical strategies.

Keywords: Nitric oxide synthases; nitric oxide chemical reactivity; nitric oxide-mediated cellular signals; neurodegeneration; neuroprotection and survival; nitric oxide synthase inhibitors; nitric oxide donors.

1. BIOCHEMISTRY OF NITRIC OXIDE SYNTHASE AND NITRIC OXIDE-RELATED SPECIES

Nitric oxide (NO) is produced by nitric oxide synthases (NOS), a family of enzymes that catalyze the two step oxidation of L-Arginine (L-Arg) to L-citrulline (L-Cit), via N^{ω}-hydroxy-L-Arginine (NHA) with final formation of NO. According to a widely shared, but not unanimously accepted scheme, the reaction flows as follows [1, 2].

$$\text{L-Arg} + O_2 + \text{NADPH} + H^+ \rightarrow \text{NHA} + \text{NADP}^+ + H_2O$$



$$\text{NHA} + O_2 + 1/2\ (\text{NADPH} + H^+) \rightarrow \text{L-Cit} + \bullet\text{NO} + 1/2\ \text{NADP}^+ + H_2O$$

Three NOS isoforms, encoded by genes located on different chromosomes, have been identified [3]. The three isoforms are similar in the overall structure and are from ~50 to ~60% homologous to each other. Two NOS isoforms, nNOS and eNOS (neuronal NOS or NOS1 and endothelial NOS or NOS3 respectively, named from the tissues where they were first identified), are constitutively expressed in different tissues and are activated by Ca^{2+}. The third isoform, iNOS (inducible NOS or NOS2), is expressed by macrophages and microglia in response to inflammatory stimuli and is not Ca^{2+} dependent [4]. Furthermore, several NOS isoforms generated by alternative splicing have been identified [5, 6, 7].

1.1 Nitric Oxide Synthases

NOS enzymes ("Fig. (**1**)") are composed of an amino-terminal oxygenase domain (NOSox) and a carboxy-terminal reductase domain (NOSred). The two domains are separated by a calmodulin (CaM) binding motif responsible for the activation of the enzyme. The NOSred contains binding sites for the redox cofactors NADPH, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) and ensures electron supply to the oxygenase domain. NOSred is similar to another flavin-*heme*-containing enzyme, cytochrome P450 reductase, and maintains some of the key features of this protein. On this basis, a three sub-domain architecture can be predicted for NOSred, constituted of the three binding sub-domains for NADPH, FAD and FMN, arranged in sequence from the C- to the N-terminal respectively [9, 10]. The oxygenase domain, the catalytic site where NO is produced, binds L-Arg, (6R)-tetrahydrobiopterin (H_4B) and a cysteinyl thiolate-ligated *heme* group (Cys194 in murine iNOS). During NO synthesis, electrons are transferred from NADPH to the flavins and from flavins to the *heme* group where iron reduction occurs. Reduced ferrous *heme* can then bind and activate O_2 for substrate oxygenation and NO synthesis. Catalytically active NOS are homodimers, only NOSox from each monomer being important for dimerization, although some data indicate that additional interactions from reductase domain are possible in nNOS and eNOS [11]. Deletion analysis on iNOSox has led to the identification of a segment at the N-terminal region (residues 66-114 in murine iNOS), necessary for dimer stability, catalysis, H_4B and L-Arg binding [12]. A conserved glutamate (Glu450 in murine iNOS) located downstream to the CaM binding

*Address correspondence to this author at the Department of Biology, University of Bologna, Via Selmi 3, 40126 Bologna, Italy; Tel: +39 051 2094134; Fax: +39 051 251208; E-mail: acontest@alma.unibo.it

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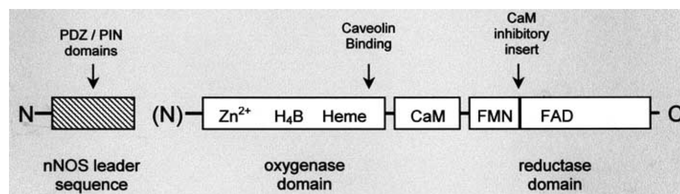


Fig. (1). Schematic general structure of NOS enzymes, including separately the nNOS leader sequence, showing the sites of binding of co-factors and regulators.

motif also appears to be crucial, as mutation to alanine (Ala) completely abolishes dimerization [13]. Dimerization occurs through a two-stage process. The NOSred assembles first with FAD, FMN and CaM, followed by incorporation of *heme* in NOSox, dimerization of monomers and incorporation of H_4B [9]. Heme incorporation seems to be necessary for dimerization, L-Arg and H_4B binding [14, 15]. Heterodimers composed of a mutated and a wild-type iNOS monomers or two monomers with dissimilar mutations, are active in almost all cases. An exception is the *heme*-defective mutant Cys194 to Ala, that was found to be unable to form homodimers and was only able to generate inactive heterodimers. It was, therefore, concluded that binding of at least one *heme* group to NOS is necessary for dimerization while two *heme* groups are required for catalysis [16]. Data from iNOS heterodimer studies utilizing mutated monomers, also revealed that electrons from one NOSred are transferred only to the NOSox of the adjacent subunit and this is sufficient to support normal rates of catalysis [17, 18].

Crystal structure of monomeric N-terminal truncated murine iNOSox was first resolved. The central core region appears to be formed by overlapping winged β-sheets, arranged in a half moon-like structure with the *heme* in the center, and flaking regions mainly having α-helix structure [2]. Crystal structures of murine dimeric iNOSox [19] and rat eNOSox [20] have been determined. The overall topology and structure of the two isoforms is similar. In the dimeric complex, residues from both sides of interacting NOSox form an extensive dimer interface (more pronounced for eNOS than iNOS). The dimer interface creates two identical H_4B binding sites and participates in structuring both the substrate binding site and active channel, according to the observation that dimer assembly is essential for H_4B incorporation, L-Arg binding and NO synthesis. Dimerization also exposes a *heme* edge into the solvent-accessible side of the protein. Residues in this area are highly conserved among NOS isoforms and this has been proposed as the docking site for NOSred interaction and electron transfer [19, 21]. L-Arg is kept close to the *heme* group and to H_4B by a network of hydrogen bonds. The amino group of L-Arg and the pterin ring both interact with a *heme* propionate group while L-Arg guanidine group is located above the *heme* with the terminal nitrogen 3.8 è away from the iron. The other two guanidine nitrogens are hydrogen-bonded to the side chain carboxyl group of a conserved glutamate (Glu371 in murine iNOS). Mutagenesis of this conserved residue completely abolishes L-Arg binding [22, 23]. H_4B also interacts with residues from both monomers and thus creates a link between the two subunits, stabilizing dimer structure. The overall structure of the core oxygenase domain is not affected by dimerization. A controversial point concerns the structure of the N-terminal region. In the iNOS structure a N-terminal β-hairpin hook interacts primarily with the adjacent subunit and an inter-subunit disulfide bond is present across the dimer interface between a symmetrical pair of conserved cysteine residues (Cys109 in murine iNOS) located on each interacting subunit. In the rat eNOS structure, the N-terminal hook Cys101 (murine iNOS 109) and the conserved Cys96 (murine iNOS 104) residues from interacting subunits, symmetrically tetracoordinate a zinc atom at the dimer interface. It was eventually found that both structures were possible for murine iNOS, as well as for human eNOS and iNOS, each isoform being able to arrange the four cysteines in both the disulfide and zinc-tetracoordinate conformation [21, 24, 25]. The overall structure of the protein, except the cysteine surrounding region, is unchanged in each conformation and the zinc atom appears to be too distant from the active site to directly support catalysis. The conserved Cys-$(X)_4$-Cys motif is located at a crucial point near the H_4B binding segment, at the dimer interface. Crystallographic data have highlighted conformational differences in this region, between the zinc-bound and free forms, that might influence both pterin binding and dimer stability and, therefore, affect enzymatic activity. This finding supports a possible role for the N-terminal region in regulating NOS activity *in vivo*. On this basis, it opens the question of whether both forms are present in cells and what their relative biological role and function are. From crystallographic data, it has been proposed [25] that in the zinc-bound form, dimer interactions are stabilized, while in the zinc-free form conformational changes weaken pterin binding. The same authors proposed that the positively charged surface around the zinc-tetracoordination site could be the docking site for the predicted highly electronegative surface of FMN reductase sub-domain [20]. Other reports have suggested that in the zinc-free form the β-hairpin hook, interacting mainly with the C-terminal of the adjacent subunit, could stabilize the dimer. Furthermore, no differences were reported in the H_4B binding site of the alternative structures [21]. This view is also supported by data from deletion and mutagenesis studies on N-terminal region of iNOSox dimers, indicating that mutations

affecting stability of the disulfide bound conformation also affect dimerization, H_4B stability and NO synthesis. Mutagenesis of Cys109 to Ala did not affect dimer stability or NO production but decreases H_4B binding [26]. Biochemical data utilizing dimeric B_4H-free nNOSox, have shown that cofactor incorporation, L-Arg binding and shift of *heme* iron to its high spin state, is dependent upon thiol reduction [27]. When B_4H-free, full-length iNOS was used, H_4B binding and full recovery of catalytic activity were obtained only in the presence of glutathione (GSH). The zinc content was estimated to be one atom per dimer and was not affected by H_4B incorporation [28]. Zinc content was approximately the same in purified NOS from pig brain and recombinant nNOS, indicating that this might be the preferential conformation. Furthermore, zinc release with chemical agents only affected dimer stability under denaturating conditions, while enzyme activity was preserved [29]. The non crucial role for zinc in catalysis was also confirmed by the use of Cys331 (corresponding to murine iNOS Cys109) mutant of rat nNOS, which is unable to bind zinc [30]. In view of a possible role for the cysteine switch in NOS regulation *in vivo*, it must be said that many cellular physiological and pathological processes could also affect the disulfide-bond/zinc-tetracoordinate balance. The redox state of the cell and the nitrosylation of conserved cysteines could regulate disulfide breakdown or zinc release from the dimer. A recent paper reports that incubation of purified eNOS with peroxynitrite ($ONOO^-$) causes zinc release and disruption of SDS-resistant dimers. Furthermore, $ONOO^-$ treatment caused uncoupling of eNOS activity with a decreased L-citrulline synthesis and a parallel increase in NADPH consumption a situation that may account for superoxide ion (O_2^-) production (see below) [31].

1.2 Role of Co-Factor and Mechanism of Reaction

H_4B is a necessary cofactor for NO synthesis but its exact role has not yet been determined. Both allosteric modulation and redox balance may account for H_4B critical contribution to the NOS reaction. Allosteric effects of H_4B include stabilization of the dimer once formed or, in the case of iNOS, promotion of dimer assembly [32, 33], increase in L-Arg binding in a cooperative manner (while H_4B binding is non-cooperative) and, synergically with L-Arg, stabilization of high-spin *heme* iron [34]. In addition, H_4B was found to influence the *heme* ferrous-dioxy intermediate reactivity [35] and to promote *heme*-dependent NADPH oxidation in response to substrate [36]. Data about pterin chemical requirement to support iNOS function, show that a fully-reduced pterin ring is necessary for NO synthesis and NADPH consumption in response to substrate. Other allosteric functions, such as promoting iNOS assembly, stabilizing the dimer structure and high-spin *heme* iron, also involve dihydrobiopterins. Different substitutions of H_4B ring side chain in position six, support both NO synthesis and *heme* reduction in response to substrate, indicating a non-absolute structural requirement for this position during catalysis, while allosteric stabilization and binding affinity are differently affected by substitutions [36]. Besides, these allosteric effects are also produced by the potent, fully-reduced NOS inhibitor, 4-amino-H_4B, suggesting that the natural cofactor has an additional catalytic function [37, 38]. Conversely, crystal structures of iNOSox dimer binding either H_4B, 4-amino-H_4B or inactive dihydrobiopterin, indicate a similar binding interaction and suggest that conformational differences are not responsible for pterin inactivity [39]. Several biochemical data support a direct role of H_4B as electron donor to the *heme* group, at least in the first step of the reaction [40, 41, 42, 43, 44]. Accordingly, H_4B radical species were detected during NHA synthesis from L-Arg by different groups, but only a small amount accumulates in the second step of the reaction [45, 46, 47]. Eventually H_4B-free iNOS was found to be able to oxidize NHA, but not L-Arg [48], while nNOS supported both steps of the reaction [49]. However, under this condition the reaction appeared to be uncoupled from NADPH consumption, nitrite and nitrate being generated without NO formation. During the reaction, a ferrous *heme* -NO complex was formed, instead of ferric *heme*-NO complex usually seen with pterin loaded nNOS, thus resulting in nitroxyl anion (NO^-) formation which rapidly generates nitrite and nitrate. Therefore, H_4B appears to be necessary in both steps of the reaction: coupling NADPH oxidation to product formation and bringing to the generation of the correct products. Other insights on H_4B function as redox cofactor came from mutation studies of a conserved tryptophan (Trp457 in murine iNOS) located in the H_4B binding site. Kinetics of H_4B radical rates of appearance and decay were affected by mutations when compared with wild-type enzyme. Mutants had slower H_4B radical formation and faster decay associated with a decrease in L-Arg hydroxylation. Crystal structure of the mutants indicates that the Trp457 aromatic side chain may be involved in stabilization of the pterin radical [50, 51, 52].

NOS are one of the two families of mammalian enzymes which use biopterins as cofactors, the other being aromatic amino acid hydroxylases, but the non-*heme* metal ion, pterin-dependent oxygen activation mechanism of these second enzymes [53, 54] are different from NOS reactions [43, 55]. During the first step of reaction, NOS performs the two electron aerobic oxidation of L-Arg to NHA by consuming two NADPH-derived reducing equivalents [56, 57]. There is agreement in the literature in indicating that oxygen activation in this first step of NOS reaction proceedes through a stepwise *heme* reduction in part similar to that of cytochrome P450 oxygenases. Ferric *heme* is first reduced by an electron provided by the reductase domain to allow ferrous-dioxigen (I) intermediate formation [35, 49, 58]. A second electron reduces the ferrous-dioxygen intermediate to a ferric-peroxo (II) intermediate, which leads to the final iron-oxo (III) species, analogs to that of cytochrome P450 reactions ("Fig. (**2**)") [44, 45, 47]. The second electron in the reaction can be provided either by the reductase domain or by H_4B, through radical formation and subsequent H_4B regeneration by an electron from the reductase domain. Indeed, this second possibility seems to be well accepted, although pterin radical reduction back to H_4B has not been investigated. The reason for the necessity of H_4B to donate an electron to the ferrous-dioxygen intermediate might be that this occurs faster than from the reductase domain and ensures coupling of oxygen activation to L-Arg hydroxylation, before the ferrous-dioxygen intermediate decays into superoxide ion and ferric *heme* [47, 59]. In the second step of the reaction, the aerobic oxidation

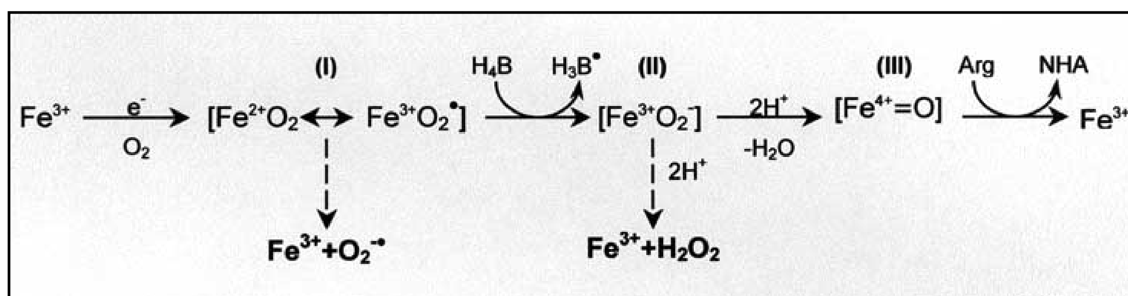


Fig. (2). Representation of the three principal steps of the first part of the catalytic reaction carried on by NOS.

of NHA to NO and citrulline is achieved by consuming only one reducing equivalent from NADPH [57, 60]. Several proposals have been advanced to account for the unusual chemistry of this reaction [44, 45, 48, 49, 52, 59, 61- 63].

1. 3 Nitric Oxide Synthase and Radical Generation

The nature of the NOS reaction products was also a matter of debate. It was suggested that NO^- was the initial product from NOS reaction, subsequently converted to NO by the action of superoxide dismutase (SOD) [64, 65]. Eventually, however direct NO production from NOS in the absence of SOD was demonstrated [66]. Heme-oxy intermediate during catalysis are unstable and can release O_2^- or H_2O_2 if the substrate is not present to accept activated oxygen "Fig. (**2**)". Furthermore, O_2^- may dismutate to H_2O_2 in solution and delivery of electrons to the *heme* must occur at a sufficient rate and with the appropriate timeliness, in order to ensure maximally coupled production of NO and to minimize uncoupled reactions [67].

NOS has been known to generate O_2^- and H_2O_2 from over a decade, but mechanism and regulation has been only recently elucidated [68, 69]. Superoxide production has directly been detected by EPR spin trapping experiments for the three NOS isoforms. Purified NOS in the presence of H_4B, produces O_2^- when catalysis occurs at low L-Arg concentration, the process being Ca^{2+}/CaM dependent for nNOS and eNOS and being inhibited by addition of L-Arg [70, 71, 72]. Enhanced production of O_2^- at the expense of NO was also detected by spin trapping in different cell systems upon L-Arg depletion: nNOS transfected cells after intracellular Ca^{2+} increase [70], activated macrophages expressing iNOS [73] and cultured cerebellar granule neurons after N-methyl-D-aspartate (NMDA) stimulation [74, 75]. Addition of L-Arg or the specific NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) were able to abolish O_2^- production from cells in all cases. Furthermore, different reports indicate the NOS inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA) was unable to suppress O_2^- formation by NOS, thus suggesting that simple occupation of substrate binding site is not sufficient to account for inhibition of O_2^- production [68, 69, 72]. Also $ONOO^-$ generation from concomitant production of NO and O_2, and subsequent nitrotyrosine formation was reported in L-Arg depleted cells [70, 73]. An important role in controlling O_2^- production is played by H_4B. Addition of L-Arg at saturating concentration to pterin-free nNOS and eNOS, resulted in only partial inhibition of O_2^- production, while addition of H_4B to substrate-free nNOS led to a greater decrease in O_2^- production without affecting NADPH consumption. Under this condition, *heme* blockers were only able to suppress approximately 50% of O_2^- production from nNOS, indicating that oxygen reduction occurs from both the *heme* group and the flavins of the reductase domain. In contrast O_2^- production from eNOS was found to be derived only from the oxygenase domain. Together H_4B and L-Arg abolished O_2^- production and enhanced citrulline synthesis. This indicates that O_2^- production was suppressed at both nNOS *heme* groups and the reductase domain and that H_4B binding couples the electron flow from the flavins to the *heme* group preventing electron leakage from the reductase domain. [76, 77]. Conversely, recent data from spin trapping experiments with H_4B-free and H_4B-bound nNOS in the absence of substrate, have demonstrated that H_4B was able to promote H_2O_2 formation at the expenses of O_2^-. This can be explained by the fact that in the absence of L-Arg, donation of one electron by H_4B to the ferrous-dioxygen intermediate allows the formation of a ferrous-peroxo species that decays generating H_2O_2 [67]. This was also confirmed by the fact that redox inactive dihydrobiopterins were unable to decrease O_2^- production by eNOS in the absence of substrate and that oxygen reduction by H_4B-free eNOS was only slightly affected by addition of L-NAME [78]. Again, the pterin cofactor appears to be essential for NOS function, as it ensures maximal coupling of NADPH consumption to L-Arg oxidation and NO production when the substrate is present and prevents dissociation of the ferrous-dioxygen complex when L-Arg is absent. Data from cell cultures, indicate that at normal intracellular levels of L-Arg and H_4B, NOS does not produce significant O_2^- and that, if some is generated, it is probably efficiently scavenged by SOD. However, if substrate or cofactor concentrations are low, as might occur in pathological condition, both NO and O_2^- are produced and the potent cytotoxic radical $ONOO^-$ is formed

1.4 Nitric Oxide Synthase Regulation and Interactions with Other Proteins

As previously noticed, CaM binds to a recognition sequence located between NOSred and NOSox. In nNOS and eNOS, catalytic activity is dependent upon CaM binding in response to Ca^{2+} increase. On the contrary, iNOS appears to be continuously active once assembled consistent with findings that indicates that CaM binding to this isoform is essentially irreversible and does not require high Ca^{2+} levels [79, 80]. It is not clear from the literature if NO production by iNOS is completely independent on Ca^{2+} levels. It has

been reported that in cell lysates iNOS half-maximal activity (EC50) occurs at Ca^{2+} concentration near to zero [81], but also that addition of EGTA to purified iNOS resulted in decreased activity [82]. In iNOS, a region between CaM and FMN sub-domains appears to be responsible, together with the canonical CaM binding sequence, for the CaM high affinity binding characteristic of this isoform [81, 83]. In contrast, eNOS and nNOS binding to CaM is negatively regulated by a ~40 amino acid insertion, located within the FMN sub-domain and absent in iNOS. This insert is responsible for CaM displacement at low Ca^{2+} concentration but it also appears to have a role as electron gate from NOSred to the *heme* in the absence of CaM binding [84, 85, 86]. CaM binding activates NOS by triggering electron transfer through the flavins and from flavins to the *heme* group, thus mainly influencing the reductase domain without affecting properties of the oxygenase domain. The exact mechanism and structural features are largely unknown, but it has been proposed that CaM binding allows proper alignment between reductase and oxygenase domains for electron transfer [87, 88, 89]. Furthermore, a control element, constituted of a ~35-40 residues tail, present in NOSred C-terminal and absent in the homologous cytochrome P450 reductase, appears to negatively regulate electron transfer between flavins in the absence of CaM binding. C-terminal tail truncated eNOS and nNOS, were able to produce NO in the absence of CaM, while NO production was decreased by CaM binding with respect to full-length enzymes [90]. Conversely mutagenesis studies on a conserved phenylalanine (Phe1395 in rat nNOS), located adjacent to the C-terminal tail, demonstrate that this residue is important as it inhibits electron transfer through the flavins in the absence of CaM and additionally promotes electron flow to the *heme* and couples NADPH consumption to NO production upon CaM binding [91].

NOS have been reported to interact with many cellular proteins regulating both activity and targeting of the enzyme. All NOS isoforms contain a binding sequence for caveolin family of scaffolding proteins. Interaction with caveolins is thought to target NOS to caveolae membranes, a cellular specialized signal-transducing domain. Purified nNOS was found to preferentially interact with caveolin-3 while eNOS with caveolin-1. In all cases, caveolin binding determined inhibition of NO synthesis, which was reverted upon CaM binding [11, 92, 93, 94]. Much of the work on NOS-caveolins interaction has been done with eNOS. The endothelial isoform was found to be associated with the Golgi complex and caveolae of endothelial cells [95, 96, 97]. Interaction with caveolin targets eNOS to caveolae in an inactive form. Intracellular calcium increase triggers CaM binding to eNOS, with concurrent release from the caveolin complex and activation of NO synthesis. As Ca^{2+} concentration goes back to basal levels, the inhibitory association with caveolins and target to caveolae are restored [98, 99]. Post-transcriptional modifications, such as myristoylation and palmitoylation seem also important for eNOS targeting and activity. The fatty acylation site located in eNOS N-terminal, appears to be sufficient for efficiently targeting eNOS at the Golgi complex and cellular membranes. Mutation of the fatty acylation site caused mislocalization and impaired NO production from eNOS [97, 100].

Activity of constitutive NOS isoforms is regulated by multiple protein interaction. Interaction of Hsp90 with nNOS and eNOS causes an increase in enzyme activity by enhancing CaM binding [92, 101, 102]. Furthermore, Hsp90 interaction was also found to inhibit both eNOS and nNOS O_2^- production at low L-Arg concentration while enhancing NO synthesis in the same conditions [103, 104]. Both nNOS and eNOS have been reported to interact with the bradykinin receptor B2. This interaction has an inhibitory effect on NOS enzymatic activity. Upon receptor activation NOS is released from the complex and becomes active. It was proposed that bradykinin receptor binding to NOS inhibits flavin to *heme* electron transfer [105, 106].

The nNOS polypeptide displays the unique feature, among NOS isoforms, of an amino-terminal leader sequence, upstream of the oxygenase domain ("Fig. (**1**)"), containing a PDZ binding motif that is responsible for postsynaptic localization in neurons through interaction with postsynaptic density proteins 95 and 93 (PSD95 and PSD93) [107]. This post-synaptic organization is of great importance for the well described functional link between glutamatergic synaptic activity and nNOS catalytic production of NO. The close vicinity between NMDA receptor and nNOS, is suggested by several biochemical and immunohistochemical ultrastructural data showing that the largest amount of nNOS is localized in close proximity of post-synaptic specializations, where a high density of NMDA receptors is also found [107]. PSD proteins possess a modular structure comprising three PDZ domains that can bind and held together nNOS and the carboxy-terminal tail of NMDA receptor subunits, thus forming a physically coupled macromolecular complex. This physical coupling has its functional counterpart in the rapid activation of nNOS catalytic activity through the calcium influx operated by the NMDA receptor channel. This peculiar arrangement allows to conclude that several effects played by NO on synaptic plasticity occur in close association with the NMDA receptor-mediated glutamatergic neurotransmission [107]. The PDZ domain also accounts for nNOS interaction with alpha-1-syntrophin, a dystrophin-associated protein, responsible for nNOS targeting to sarcolemma of skeletal muscle [107]. The association of nNOS to post synaptic density is also negatively regulated by a protein named CAPON, which competes with PSD95 for interaction with nNOS. Overexpression of CAPON results in a loss of PSD95/nNOS complexes in transfected cells [108]. It was also recently reported that nNOS forms a ternary complex with CAPON and synapsin, supporting its presynaptic localization [109].

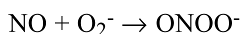
The plasma membrane Ca^{2+}/CaM-dependent calcium ATPase (PMCA), which contributes to intracellular calcium homeostasis, also binds to nNOS through the PDZ domain. Binding of PMCA to nNOS results in decreased catalytic activity, while PDZ-defective nNOS was not regulated by PMCA [110]. The leader sequence also contains a binding site for a highly conserved protein named PIN (protein inhibitor of NOS). Binding of PIN causes destabilization of nNOS dimer [111, 112].

NOS can also be phosphorylated on multiple residues, leading to different effects on the enzymatic activity. Endothelial NOS was found to be phosphorylated on a

conserved serine residue (Ser1177 in human eNOS), located in the C-terminal, by the kinases Akt and PKA. Phosphorylation of Ser1177 leads to an increase in NO production and a decrease in Ca^{2+} dependency of the enzyme [113, 114, 115]. It was proposed that this effect was due to an increased rate of electron flow through the reductase domain and a decrease in CaM dissociation at low Ca^{2+} concentration [116]. In addition, an also conserved threonine residue (Thr495 in human eNOS) located in the CaM binding motif, has been shown to be important for NOS regulation. Phosporylation of Thr495 had an inhibitory effect on catalytic activity [117], whereas dephosphorylation enhanced CaM binding to eNOS [118]. These two phosphorylation sites appear to play opposite roles in eNOS regulation. In fact, growth factor stimulation of endothelial cells caused phosporylation of Ser1177 and dephosphorylation of Thr495, with the final result of increasing eNOS activity. The same study also demonstrated that phosphorylation/dephosporylation of conserved residues was mediated by PKA, PKC and the two phosphatases PP1 and PP2A [119]. Similar effects on Ser/Thr phosphorylation state were obtained upon bradykinin stimulation [120]. Neuronal NOS was found to be phosphorylated on a serine residue (Ser847 in rat nNOS) by CaMKII, with the result of inhibiting catalytic activity both *in vitro* and in co-transfection experiments [121, 122].

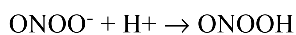
1.5 Nitric Oxide-Related Species and Their Reactions

Nitric oxide-related species may be present in living organisms in three different redox forms. In addition to the radical form $NO^{\bullet}$, species having an additional electron (NO^-, nitroxyl anion) or having one electron less (NO^+, nitrosonium ion) may be produced by cells. These different species possess different stability, ability to diffuse through biological structures and, most important, different chemical reactivity. The favored reactant for NO is constituted by the superoxide anion O_2^-, a side product of oxidative metabolism continuously produced by cells. It is, indeed, evaluated that 20% of total bodily oxygen consumption in resting conditions, takes place in the brain and that about 2% of this oxygen is released in the form of partially reduced, reactive oxygen species (ROS) [123]. In the presence of high NO concentration, the O_2^- scavenging activity of superoxide dismutases may be overcomed with the formation of peroxynitrite ion according to the reaction:

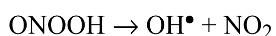


$NO + O_2^- \rightarrow ONOO^-$

Peroxynitrite is a relatively unstable compound and an agent of oxidative damage to proteins, lipids, nucleic acids and key cellular organelles, such as mitochondria [124, 125]. By protonation, peroxynitrite gives rise to peroxynitrous acid which, through intermediate isomerization reactions [126], decomposes producing a very toxic and highly reactive ROS, the hydroxyl radical $OH^{\bullet}$:



$ONOO^- + H+ \rightarrow ONOOH$



$ONOOH \rightarrow OH^{\bullet} + NO_2$

NO also reacts with molecular oxygen through a two-step reaction bringing to formation of N_2O_3, which may be hydrolized by reacting with H_2O or may act as an NO-related species donor. NO-related species easily react with several functional groups of organic molecules, for instance aminic, thiolic and hydroxylic residues, transferring to them NO^+ (a process chemically identified with the term nitrosation), NO_2^+ (a process called nitration) or directly adding the NO radical without changing substrate charge (a process called nitrosylation) [126]. Some of the relevant biological consequences of the modifications caused by NO-related species to biomolecules will be considered later in this review.

While N_2O_3 is the main species involved in nitrosative reactions, pro-oxidant NO-related species (NO, $ONOO^-$, NO_2, HNO) potentially contribute to oxidative damage. However, these same chemical entities may actually bear an antioxidant function, as they are able to scavenge more toxic oxygen radicals, such as superoxide. Furthermore, these NO-related species counteract the formation and the accumulation of the much more dangerous hydroxyl radical formed through the Fenton reaction (the reaction of ferrous ions with hydrogen peroxide to give Fe^3 and $OH^{\bullet}$). A further way to counteract oxidative stress is the scavenging of the thiyl radical $GS^{\bullet}$, resulting from excessive oxidative burden on GSH, which is converted to S-nitrosoglutathione (GSNO), further acting as antioxidant [127]. NO-related species may also exert an effective antioxidant function by competing with potential targets of ROS action. This is the case for lipid peroxidation proceeding through a chain reaction giving rise to a large amount of oxy-and peroxy- lipid radicals. NO-related species block the propagation of the chain reaction producing nitrosylated lipids, of the LOONO general structure, with lower reactive potential.

Nitric oxide was recognized as an intercellular messenger molecule in the brain about 15 years ago [128]. Its role as a new type of diffusible neurotransmitter/neuromodulator, able to activate several signaling pathways and involved in synaptic plasticity and development has been corroborated by many researches during the last decade [see 129, 130, 131 for recent reviews]. Even greater focus has been centered, during the same years, on the neurotoxic role of NO [see 129, 132, 133 for recent reviews] and actually, if one makes a review of recent literature, it clearly appears that more neuroscientists consider the neuropathological actions of NO to prevail on its neuroprotective and physiological actions. In the light of its high chemical reactivity, it is not surprising that multiple cellular and molecular targets have been identified as mediators of NO effects. The first identified, and probably the most important, cellular target of NO is the soluble form of guanylate cyclase, whose product, cGMP, is further responsible for the activation of protein kinase G (PKG) and several other cellular effectors [134, 135, 136, 137]. Activation of the guanylate cyclase *heme* group occurs through a pentacoordinate ferrous-nitrosyl complex [138]. It has become more and more clear, however, that NO actions are multifarious in the cell. Switching and regulation of proteins through S-nitrosylation is now recognized as a primary mechanism to control protein function [139]. Accordingly, this NO-related process is presently considered to represent one of the most important ways through which NO regulates cell function. Besides guanylate cyclase activation and protein nitrosylation, NO has been demonstrated to be able to operate through several other mechanisms [129, 132, 140-142]. It will not be surprising to find in the near future new cellular targets for

NO and new ways of regulating complex physiological functions or of disregulating them in pathological conditions.

2. PHYSIOLOGICAL ROLES OF NITRIC OXIDE

NO is a versatile diffusible messenger implicated in various physiological functions, ranging from dilatation of blood vessels and muscle relaxation to immune responses and potentiation of synaptic transmission [134]. Although NO was first identified as endothelium-derived relaxing factor, it is now clear that the brain is a primary source for its production in the body. NO has been implicated in developmental neural plasticity [131, 143, 144] as well as in hippocampal long-term potentiation (LTP), a long-lasting form of synaptic plasticity related to some types of learning and memory [145, 146]. Data accumulated during the last decade indicate that NO participates in the regulation of neurotransmission in the central nervous system [147-149], modulating neuronal release of neurotransmitters under *in vitro* [150] and *in vivo* [149] conditions. Among other functions, NO participates in the regulation of the monoamine-mediated neurotransmission [147], can inhibit the uptake of dopamine, noradrenaline, and 5-HT [151, 152, 153] and is able to enhance the release of acetylcholine from basal forebrain neurons [149]. In addition to its function as neurotransmitter regulator, NO itself is an important messenger with neurotransmitter-like function. However, NO differs from classical neurotransmitters, in that it is not stored in synaptic vesicles and its action is not mediated by specific membrane receptors. The NO molecule mainly exerts its effects by enhancing cGMP production or by direct action on several proteins including ionic channels. Recent studies have shown that the activation of guanylate cyclase, either through applied NO, or with physiological NOS stimulation, increased neuronal firing [154]. NO modulates stimulus-evoked excitatory field potentials, EPSPs and firing rates recorded from single neurons in almost all brain regions and spinal cord [for review see 155]. Thus, the nitric oxide/cGMP cascade generates a short-term enhancement of release in nerve terminals [156].

NO, through cGMP pathway, has long been suspected to play a role in synaptic plasticity [157], acting as a retrograde messenger during LTP in the CA1 region of hippocampus. NO produced in the postsynaptic neuron, diffuses and travels through the extracellular space, directly acting in the presynaptic neuron, enhancing neurotransmitter release and thus potentiating the synaptic response [158]. Studies with NOS inhibitors suggested a role for NO in some, but not all, forms of hippocampal LTP [159]. These differences were partially explained by a recent study [160] using NOS mutant mice. LTP was normal in mice knockout for nNOS and also normal in mice knockout for eNOS, but LTP in stratum radiatum of CA1 region was significantly impaired in double knockout mice ($nNOS^-/eNOS^-$). These results provide genetic evidence that NOS is involved in LTP in stratum radiatum and suggest that the neuronal and endothelial forms can compensate for each other in mice with a single deletion. It may be expected that blocking a retrograde messenger involved in activity-dependent synaptic plasticity would affect learning. Indeed, NOS inhibitors reduce the ability of experimental animals to perform several learning tasks, supporting the role of NO in mechanisms of hippocampal synaptic plasticity associated with the memory consolidation [157, 158]. Many of the diverse synaptic functions of NO in the brain are intrinsically linked to NMDA receptors: nNOS is functionally coupled with Ca^{2+} influx through NMDA-type glutamate receptors and Ca^{2+} influx appears to help targeting of nNOS at the postsynaptic density [107, 134, 161]. Recent evidence indicates that NO is an important player in the programme of brain development, by influencing the escape from the proliferative state and the acquisition of the differentiated phenotype by neurons. NOS expression and NO synthesis are strongly induced in cultured cells treated with growth factors, and there is increasing evidence that many of the pleiotropic effects of growth factors may be mediated through NO [162-165]. In particular, NO was shown to be crucial for differentiation of cultured neuronal cells. In PC12 cells, for instance, increase in nNOS expression and activity occurs in parallel with decreased proliferation and acquisition of neuron-like phenotype [162, 164]. The importance of NO for PC12 differentiation, was further stressed by the fact that transfection with a dominant negative mutant nNOS expression vector, significantly counteracted NGF-induced differentiation [166]. Moreover, NOS inhibitors have been shown to attenuate NGF-mediated increase in choline acetyltransferase expression in PC12 cells [167]. In human neuroblastoma cells, differentiation induced by treatment with TNF-α [164] or injection with HIV-1 [168] demonstrated a dependence upon iNOS activity [164]. Whereas Ghigo and co-workers [169] have shown that neuroblastoma SK-N-BE cell differentiation was not induced by an NO donor, nor did treatment with L-NAME inhibit retinoic acid-induced differentiation, we have recently observed that overexpression of nNOS speeds up differentiation of neuroblastoma cells (Ciani *et al.*, manuscript in preparation). We established a more direct link between neuroblastoma cell differentiation and NO, by showing that an nNOS overexpressing clone of SK-N-BE cells responds much more quickly than parental cells to the differentiative action of retinoic acid. Furthermore, we also observed that the process of differentiation towards a neuronal phenotype was accompanied in this clone by decreased proliferation, an effect clearly related to NO production, since it was blocked by NOS inhibition (Ciani *et al.*, manuscript in preparation). Accordingly, it was shown that endogenous and exogenous NO can decrease proliferation in a neuroblastoma cell line (NB69) that naturally expresses the EGF receptor, through inhibition of the mitogenic signal initiated by EGF [170]. Studies on *Drosophila* have been particularly useful in disclosing the role of NO in organ development and tissue differentiation [171]. NO is produced at high levels in imaginal discs at the end of the larval stage and acts as an essential negative regulator of cell proliferation. Manipulation of NOS activity in the developing larvae, affected the size of the adult organs, its inhibition resulting in surplus cell proliferation with excessive growth [172, 173], whereas ectopic expression of NOS having the opposite effect [173]. The morphogenic regulation by NO can further be appreciated at later stages of development, when NO affects the formation of retinal projection pattern. It has been demonstrated that inhibition of the NO/cGMP pathway leads to overgrowth of

retinal axons [174] and that diminished soluble guanylate cyclase activity in the visual system during development, causes inappropriate or inadequate formation of first-order retinal synapses, leading to defects in fly visual system and in visually mediated behaviour [175]. NO synthesis was also demonstrated to be essential for the transition from proliferation to cell cycle arrest during brain development in *Xenopus* [176], as NOS inhibition yielded to larger brains with grossly perturbed organization. On the contrary, exogenous NO decreased the number of proliferating cells and the total cell number in the optic tectum. It was suggested that, in the mammalian visual system, NO released from target neurons in the superior colliculus and in the lateral geniculate body serves as a retrograde signal which feeds back on retinal afferents, influencing their growth [177].

NO may also be important for the control of cell division in the developing and adult mammalian brain. Inhibition of NOS by L-NAME significantly increased the number of BrdU labeled cells in the dentate gyrus of the hippocampus of adult rats, supporting the suggestion that NO is involved in neural progenitor cells proliferation [Waddington-Lamont *et al.*, Soc. Neurosci. Meeting, 2002, Abstr. 23-10]. However, no effects of NO on neurogenesis have been established using NOS knockout mice [131]. In addition, attempts to probe the role of NO in mammalian brain morphogenesis are complicated by the unusual complexity of NOS genes: in particular, the presence of alternative promoters and splice sites leads to the generation of multiple nNOS RNA and protein isoforms [51, 161, 178]. On the ground of multiple evidence for a role of NO in neuroprotection, it will be important to examine in more detail the role of NO in mammalian brain development by performing rigorous quantitative analysis of neurogenesis in NOS knockout mice.

3. NITRIC OXIDE AND NEURODEGENERATION

Soon after discovering NO as a mediator of neuronal physiology, its role in glutamate toxicity, and, therefore, its involvement in neuropathology, was suggested [179]. While it has been shown that NO contributes to both acute [180, 181] and chronic [182, 183, 184, 185, 186] degenerative diseases, its exact role in neuropathology is not fully understood.

3.1 Neuropathological Role of Nitric Oxide

The role of NO in brain ischemia, while highly controversial (see section 4), has been extensively studied and it has been suggested that the dual NO effect (neurodegenerative and neuroprotective) depends on which isoform produces it [187, 188, 189, 190, 191].

Alzheimer’s disease, the most common cause of dementia, is characterized by the presence of plaques of β-amyloid peptide and of neurofibrillary tangles, and by a progressive loss of neurons in several areas of the brain. In Alzheimer’s disease, the data regarding the activity and the expression of the various isoforms of NOS are contrasting [192-194] and no correlation has been found among the polymorphisms in the NOS isoform genes and the disease [195, 196]. However, *in vitro*, β-amyloid has been shown to cooperate with glutamate to induce neuronal damage through NO [197-199] and to stimulate both microglia and astrocytes to produce NO [200-202]. Recently, *in vitro* experiments have demonstrated that β-amyloid inhibits the activity of nNOS and eNOS while enhancing the expression of iNOS [203].

Parkinson’s disease is a neurological syndrome characterized by the selective degeneration of dopaminergic nigro-striatal neurons. The information on the role of NO in Parkinson’s disease comes primarily from mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which reproduces clinical, biochemical and neuropathological changes occurring in idiopathic Parkinson's disease [reviewed by 204]. It has been shown that treatments with NOS inhibitors protect striatal neurons from MPTP neurotoxicity and that mice knock-out for nNOS and iNOS are less sensitive to the neurotoxin [205-207].

Huntington’s disease is an autosomal dominant fatal neurogenetic disease caused by the abnormal expansion of a polyglutamine repeat at the N-terminal of the huntingtin protein. A role of NO in this pathology is emerging [reviewed by 208]. A decrease in nNOS expression and activity has been observed in patients and in a transgenic mouse model of Huntington’s disease [209, 210]. However, the presence of nitrotyrosine in a transgenic mouse model of Huntington’s disease [Gordiner and Deckel, Soc. Neurosci. Abstr., 2000 Meeting, *26,* 498] and the data obtained by using NOS inhibitors in non-transgenic animal models for Huntington’s disease [211] suggested a role for NO in this disease.

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterized by a progressive loss of motor neurons which leads to paralysis and death. It is suspected that glutamate-induced neurotoxicity is involved in ALS pathogenesis [reviewed by 212, 213] and this suggested that NO could play a role too. In ALS patients, motor neurons are reported to be immunoreactive to anti-nitrotyrosine antibody [214], while elevated levels of NO metabolites are present in the cerebrospinal fluid [215]. NOS reactivity was marked only in the superficial dorsal horn in controls, but in ALS tissue it was intense throughout the grey and white matter [216] and nNOS beta and nNOS gamma spliced variants, but not nNOS alpha, were upregulated in reactive astrocytes in ALS [217]. However, in a more recent study, no significant alterations have been observed in the immunoreactivity of NOS isoforms in patients with ALS [218]. ALS is mainly sporadic, but about 5-10% of cases are inherited. People with familiar ALS carry mutations in the free radical scavenging enzyme superoxide dismutase 1 (SOD-1) [219, 220], therefore transgenic mice overexpressing this human mutation have been developed to study this pathology. In these mice, nNOS immunoreactivity has been observed in the anterior horn neurons starting from the presymptomatic stage [221] and reactive astrocytes are positive for iNOS immunostaining from the early symptomatic stage to the final stages [221]. Moreover, motor neurons from mutant SOD-1 transgenic mice are more sensitive to NO-triggered cell death [222]. However, by using NOS inhibitors, as well

as by studying mice with SOD-1 mutations in nNOS null background, it was concluded that nNOS is not directly involved in ALS [223].

Multiple sclerosis is an inflammatory, demyelinating disease of the central nervous system. Tissue from multiple sclerosis patients show iNOS positive glial cells in the lesioned areas [224]. In rats with experimental allergic encephalomyelitis (EAE), a validated animal model for multiple sclerosis, during late stages, constitutive NOS activity decreases concomitantly with iNOS upregulation [225]. From other reports, however, NO seems to play a role in the recovery from EAE and in the resistance to reinduction [226].

HIV infection in the brain results in dementia, which is characterized by motor deficits, cognitive impairment and behavioral disorders. NO has been shown to be involved in HIV-associated dementia in several ways. In fact, in severe HIV-associated dementia the levels of iNOS coincided with increased expression of the HIV coat protein gp41 [227]. In rats infected with gp120 HIV protein, nNOS expression was decreased in hippocampus, but not in cortex [228]. Other data, however, suggested that the viral protein induces neuronal death through a NO-dependent mechanism [229, 230] and that infected glial cells produce large amounts of NO which is potentially toxic for neurons [231, 232].

Aging is the normal consequence of the life progress in multicellular organims and is accompanied by several physiological changes that can bring to functional deficits. One of the hypotheses developed to explain brain aging postulates that NO plays a central role in aging mechanisms [182]. Data on the activity and the expression of NOS isoforms in rodent aging brain are highly controversial. In aged rats, NOS activity was reported to be decreased in hippocampus, but not in cerebellum and cortex [133], while others noticed a decrease of nNOS in cerebellum [134]. Our own observation was that only striatum and olfactory cortex from aged rats showed a decrease in the activity and the expression of nNOS, but iNOS did not change in any of the considered areas [135]. Several other studies on aged animals have demonstrated regionally variable decrease of either catalytic activity or NOS expression/localization, with emerging discrepancies concerning the brain areas to which those alterations were located [234, 236-239]. Recently, it has been demonstrated that nNOS significantly decreases in the hippocampi from congnitively impaired aged rats only [240]. In normal human aging, NOS activity does not seem to change [241].

3.2 Mechanisms of Nitric Oxide Action in Neurodegeneration

Either a decrease in NO below the physiological level that neurons need for their survival or an excess in its production can be dangerous. In the presence of high levels of NO, there are several factors that can determine whether NO is beneficial or detrimental, such as the concentration of NO and of other reactive species, the fact that the exposure is acute or chronic, and the presence of potential targets. The targets and the mechanisms of NO neurotoxicity are only partially different from those involved in its physiological action. In fact, NO exerts its pathological role by acting on i) production of reactive nitrogenous species, free radicals release and induction of nitrosative stress, ii) S-nitrosylation of protein thiols and nitration of phenolic residues, iii) lipid peroxidation, iv) mitochondrial impairment, v) DNA damage, vi) neuronal death and vii) inflammation [reviewed by 139, 242]. All of these mode of action are at least partially common to processes related to physiological and neuroprotective or to neurodegenerative effects of NO.

Reactive Nitrogenous Species, Free Radicals and Nitrosative Stress

NO can exert its pathological role either by directly acting on its targets, or indirectly, after combining with other reactants. In conditions of high NO levels and when the cellular defense mechanisms are compromised, the indirect action of NO can result in either oxidative or nitrosative stress. NO can react with several free radical species, most noticeably with the superoxide anion (O_2^-) to give peroxynitrite (ONOO-). The superoxide anion (O_2^-) can be converted by superoxide dismutases (MnSOD and Cu/ZnSOD) to hydrogen peroxide (H_2O_2), which may be converted into the highly toxic OH$^\bullet$ [243, 244] ("Fig. (**3**)"). Several are the sources of superoxide radicals, such as cyclooxygenases, lipoxygenase, xanthine oxidase, the mitochondrial electron transport chain and the NOS itself [243, 245], when the electron transfer between NADPH and arginine becomes uncoupled [246]. In neurons, excessive NOS activation and, therefore, excessive NO production, has been observed in the case of massive calcium accumulation due to hyperactivity of glutamate neurotransmission and/or mitochondrial dysfunction [74, 247-249]. Another important source of NO in pathological brain is microglia, the resident

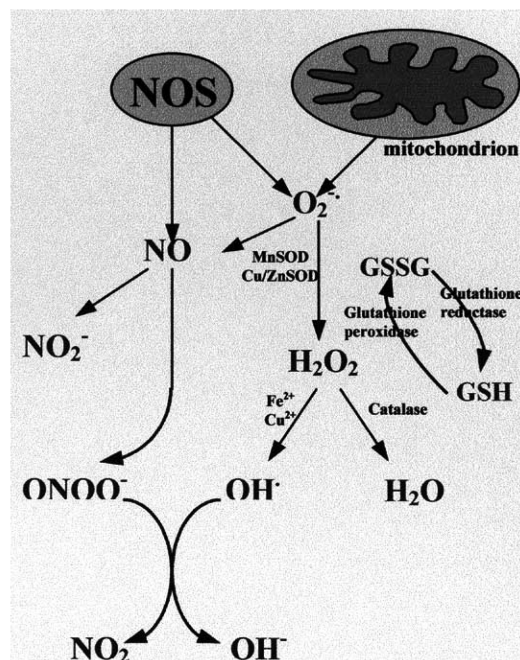


Fig. (3). Representation of some of the main cellular reactions bringing to the formation of oxygen-and nitrogen-based radicals.

macrophages of the nervous system [250], which release large amounts of NO upon inflammation [251]. Peroxynitrite acts in neurodegeneration through lipid peroxidation, DNA breakage and protein nitrosylation and nitration [reviewed by 252]. However, as outlined before, peroxynitrites too are double-faced in their action, mediating dangerous effects or scavenging more toxic radicals [126]. Peroxynitrites are known to be involved in several neurodegenerative diseases. *In vitro* experiments showed that the toxicity of β-amyloid-activated microglia towards neurons was mediated by peroxynitrites, suggesting that they play a role in Alzheimer's disease [253]. Moreover, peroxynitrites are able to oxidate dopamine and to inactivate key enzymes in the synthesis and transport of this neurotransmitter, such as tyrosine hydroxylase and dopamine transporters [254, 255], and this may be involved in the etiology of Parkinson's disease. *In vivo*, it has been observed that injection of the peroxynitrite donor 3-morpholinosydnonimine induces axonal damage, myelin alteration and demyelination similar to multiple sclerosis lesions [256] and that uric acid, a peroxynitrite scavenger, inhibits CNS inflammation, blood-CNS barrier permeability changes and tissue damage in the EAE mouse model of multiple sclerosis [257].

Protein S-Nitrosylation and Nitration

Nitric oxide and peroxynitrite can react with thiols of lysine, methionine, histidine and, especially, cysteine to form S-nitrosylated derivatives, thus altering the conformation and/or the activity of many proteins, in a way analogous to phosphorylation or acetylation [258, 259]. Several proteins can be nitrosylated [258], among them, are H-ras [260], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [258], protein kinase C [261], caspases [262, 263], tissue transglutaminase [263, 264] and NR1 and NR2 subunits of the NMDA glutamate receptor [258, 265]. S-nitrosylation differentially affects neuronal function: it can suppress caspase activity and therefore protect from apoptosis, [262, 263, 266] further it can reduce neurotoxicity related to excessive NMDA receptor activation [267], but it can also induce neuronal apoptosis through activation of matrix metalloproteinases (MMPs) [259] and inactivation of proteasome [268]. In addition to its oxidative potency, peroxynitrite can also nitrate heterocyclic compounds, like tryptophan and guanine, or phenoles, like tyrosine [269, 270]. In particular, it has been observed that nitration of tyrosines is deleterious by altering the tyrosine phosphorylation/dephosphorylation signaling and by changing the proteolytic cellular equilibrium [271, 272]. In fact, peroxynitrite has been shown to activate the MAP kinase family members p38, JNK and ERK1/2 and to induce the expression of stress genes, such as the growth arrest and DNA damage-inducible proteins (Gadd) [273, 274]. It is interesting to note that activation of JNK and/or p38 plays a crucial role in neuronal apoptosis, while ERK1/2 are protective [66]. Protein nitration has been observed in normal aging [275] as well as in several neurodegenerative diseases. The presence of nitrotyrosine has been demonstrated in Alzheimer's disease [276], in the Lewy bodies in Parkinson disease [277] and during inflammation in the EAE model [278]. In ALS, protein nitration has been shown to correlate with an increase in nNOS and eNOS expression in the anterior horn cells [279]. However, nitrotyrosination seems to give a minimal contribution to neurodegeneration in the mutant SOD1 model of ALS [280].

Lipid Peroxidation

Membrane Lipid Peroxidation (MLP) consists of a chain reaction that results in the oxidation of phospholipids to lipid hydroperoxides and in the formation of secondary diffusible products, such as aldehydes [281]. Triggering MLP in a cell, gives rise to a complicated cascade of events that brings to loss of membrane integrity, impairment of membrane-transport proteins, disruption of ion homeostasis, formation of mitochondrial permeability transition pore and, ultimately, cell death [reviewed by 282]. In addition, one of the aldehydes produced by lipid peroxidation, the 4-hydroxynonemal (HNE), is highly reactive towards membrane proteins and cytotoxic [283]. NO can both promote and inhibit lipid peroxidation. In fact, by reacting with superoxide and, therefore, generating peroxynitrite, NO can induce lipid peroxidation [246, 284]. On the other hand, NO is a potent inhibitor of the lipid chain reaction that brings to MLP [285] and, by scavenging $OH^{\bullet}$ radicals, it neutralizes a potent initiator of the reaction [286]. It has been shown that peroxynitrite-induced lipid peroxidation is involved in the pathogenic mechanism of presenilin-1 in Alzheimer's disease [287] and in motor neuron degeneration in a mouse model for ALS [288]. Contrasting effects of nitric oxide and peroxynitrite on lipid peroxidation have also been described in EAE [289].

Mitochondrial Impairment

Mitochondrial impairment has been demonstrated to be an important factor in many neurodegenerative diseases [290] and NO-related species are well known mediators of mitochondrial damage [reviewed by 291]. NO at very low concentration (nanomolar) reversibly blocks mitochondrial respiration by inhibiting cytochrome c oxidase (complex IV). In competition with oxygen, it also acts by binding to the same site of oxygen, i.e. the reduced form of *heme* a_3. This has been demonstrated in isolated mitochondria [292, 293], in synaptosomes [294] and in cells [295]. Considering that inhibition of cytochrome c oxidase by NO is more effective at low O_2 tension and that it is sensitive to the redox state of the respiratory chain, it has been suggested that low NO concentration may act as a physiological regulator of the oxygen sensitivity in respiration [296]. At higher concentration, NO also inhibits cytochrome bc_1 complex (complex III) [297]. Peroxynitrite can irreversibly inhibit mitochondrial respiration at several sites, such as NADH-ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II) and ATP synthetase (complex V), but, in contrast to NO, it is poorly active on complex IV [298]. Low levels of peroxynitrite, but only very high levels of NO, can inhibit mitochondrial respiration by blocking aconitase [299, 300]. Peroxynitrite can also cause the opening of the permeability transition pore (PTP) in mitochondrial membranes [301, 302]. PTP has been involved in both apoptosis and necrosis [303, 304]. In fact, its opening causes calcium efflux, mitochondrial swelling, depolarization and cytochrome c release [301, 302]. The release of cytochrome c and of other proteins, such as the apoptosis inducing factor (AIF) from the intermembrane space, is a central event in caspase activation and, therefore, in apoptotic cell death [303]. The way by which

peroxynitrite induces PTP opening is still controversial [291].

DNA Damage

NO-related species can also damage DNA. In fact, it has been observed that excess NO, by causing peroxynitrite formation, induced single strand breaks of DNA [304, 305]. Damaged DNA induces activation of poly(ADP-ribose)polymerase (PARP), an enzyme that is involved in DNA repair by adding ADP-ribose group to nuclear proteins. Its over-activation brings to an excessive ATP consumption and cellular energy deficit [306, 307]. DNA damage also causes an increase in cellular level of the tumor suppressor protein p53, by altering its degradation rate [308]. DNA single-strand breaks due to NO-related species have been observed in ischemic brain [309, 310], in Alzheimer's disease [311], in multiple sclerosis [312] and in motor neuron degeneration [313]. Recently, it has been observed that mitochondrial DNA is more sensitive to damage caused by NO-related species than nuclear DNA [314].

Neuronal Death

Excessive amounts of NO induces cell death either by apoptosis or necrosis, depending on specific cellular conditions and on the intensity of the insult [247]. In fact, a large NO increase causes ATP depletion, severe oxidative stress, mitochondrial swelling, disruption of ion gradients, rupture of plasma membrane and, finally, cell death through a necrotic mechanism [315, 316]. An increase in NO below the level that causes necrosis, but to a level sufficient to impair cellular repair processes, induces apoptosis. Recently, it has been shown that in primary cultures of cortical neuron, NOS activation mediated by glutamate causes transient inhibition of ATP synthesis leading to apotosis in a

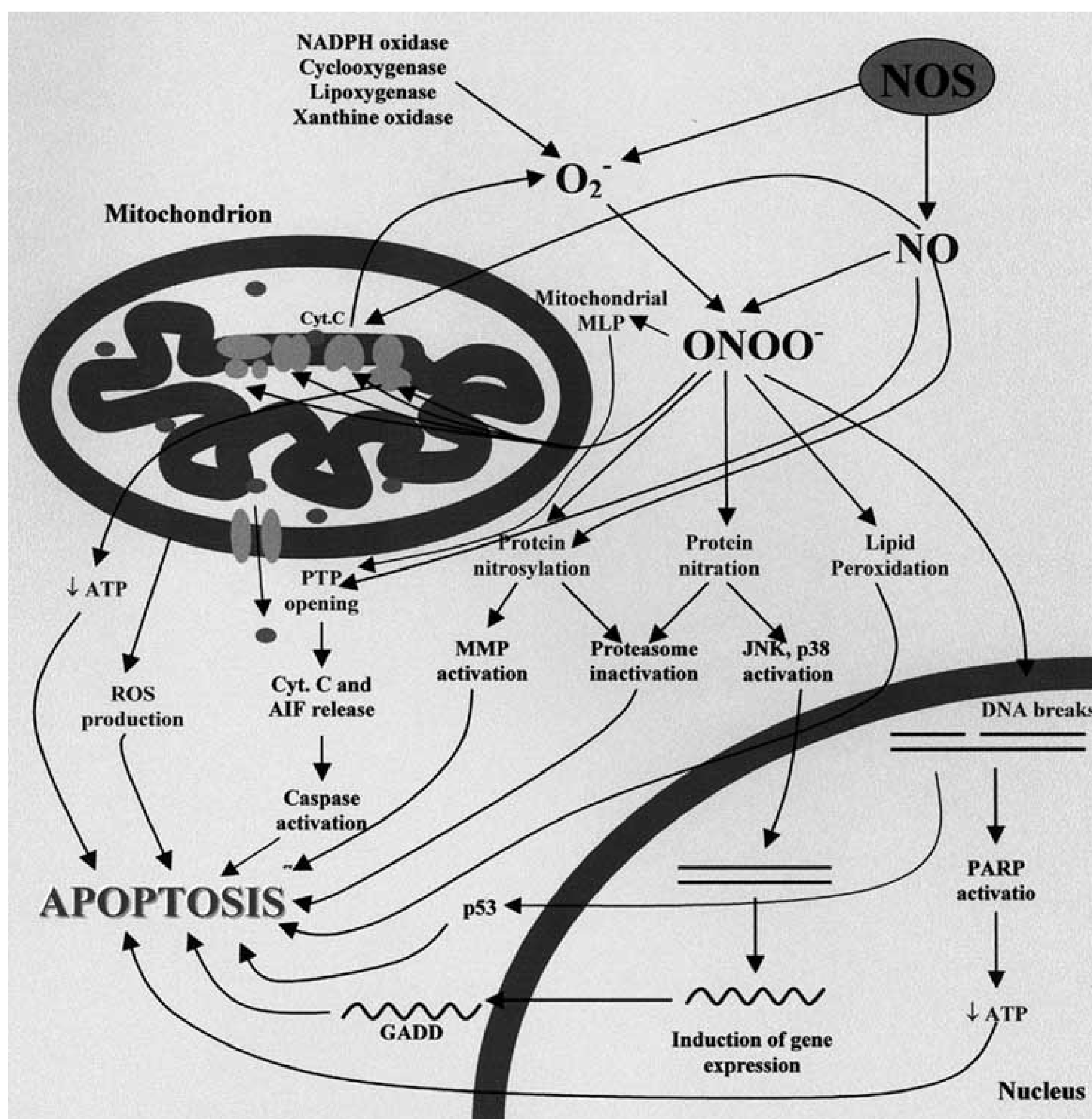


Fig. (4). Composite drawing summarizing the main cellular signaling pathways mediating the neurodegenerative effects of NO.

subpopulation of neurons, while it induces persistent block of ATP synthesis, oxidative stress and necrosis in other neurons [317]. Not only the levels of ATP, but also those of glutathione, the glucose concentration, the rate of glycolysis and the oxygen level determine how the cell die in response to NO [318, 319]. NO acts as a proapoptotic modulator through caspase activation due to opening of the mitochondrial permeability transition pore and to the consequent release of mitochondrial cytochrome c and AIF into the cytosol [301, 302, 317], through calpain activation [320], p53 upregulation [321], activation of JNK/SAPK and of p38 MAP kinase pathways [272, 232], impairment of the protein degradation systems through proteasome inactivation, [262] and alteration of the expression of apoptosis-associated factors, including Bcl-2 family proteins [323] "Fig. (**4**)". Nitric oxide-mediated apoptosis has been involved in the death of cultured motor neuron in a Cu/Zn SOD-dependent way, suggesting a role of NO in ALS [324, 325, 326]. Moreover, peroxynitrite has been suggested to mediate apoptosis in an *in vivo* model of motor neuron apoptosis [313]. It has been shown that NOS inhibitors and peroxynitrite scavengers protect PC12 cells from presenilin-1 induced apoptosis, indicating a role of NO mediated neuronal apoptosis in Alzheimer's disease [287]. *In vivo*, it has been observed that nitric oxide produced by iNOS induces apoptosis following traumatic spinal cord injury [327] while NO produced by nNOS is involved in ischemia-induced apoptosis [325, see however sections 4 and 5].

Inflammation

Inflammation is an active defense mechanism that the organism builds up against different insults. Inflammation in the brain mainly involves microglia and astrocytes [329, 330, 331]. Activated glial cells have been shown to release large amounts of NO following the induction of iNOS [332, 333]. While glial cells are resistant to high level of NO, neurons are sensitive [317, 334]. The mechanism by which glial derived NO kills neurons seems to involve excitotoxic glutamate release and impairment of mitochondria [333, 335]. Moreover, it has been observed that the effect of NO depends on neuronal maturation and on NMDA receptor expression [336].

In neurodegenerative inflammatory processes, glial derived NO can be induced by the accumulation of altered proteins, by signals from injured neurons or by alterations in the brain balance between pro- and anti-inflammatory mechanisms [reviewed by 337]. Increased glial production of NO has been observed in the aging brain [275] and in Parkinson's disease [338], Alzheimer's disease [186, 339, 340], HIV associated dementia [186, 339] multiple sclerosis [186, 341], ischemia and brain injury [342-344]. In Alzheimer's disease, reactive microglial cells have been found associated to neuritic and amyloid plaques [345]. Microglia is known to be activated by β-amyloid to increase cytokines and NO production [346, 347] and, in a co-culture system, peroxynitrites have been shown to mediate β-amyloid-induced microglia neurotoxicity [253]. Moreover, astrocytes contribute to amyloid–induced neurotoxicity in an NO-dependent way [200, 348]. In the substantia nigra from patients of Parkinson's disease, there has been observed an increased number of iNOS positive glial cells and nitro-tyrosine accumulation [277, 348]. Moreover, in the MPTP model of the disease, blockade of microglia and, consequently, of the NO production is neuroprotective for dopaminergic neurons [350, 351]. However, Liberatore *et al.* [207] showed that NO production by glial cells plays a deleterious effect on dopaminergic neurons. In the SOD-1 mutant mouse model of ALS, iNOS upregulation has been reported in glial cells [352]. Recently, it has been observed that glial cells cocultured with motor neurons respond to high level of NO through a phenotypic transformation that may contribute to motor neurons apoptosis, suggesting that NO may play a role in ALS by affecting neurons through activation of glial cells [353]. In multiple sclerosis, the role of glial cell-derived NO is controversial [reviewed by 186]. In EAE, glial activation results in iNOS induction and nitrotyrosine has been shown to colocalize with activated microglia and astrocytes [354, 355]. Moreover, *in vitro* experiments showed that microglia exert their toxic effect on oligodendrocytes through NO [356]. However, contrary to what expected, in the iNOS deficient mice, the EAE disease is more severe and prolonged [357]. In the HIV associated dementia, NO is mainly produced by microglia. In patients, it has been observed that the severity and rate of progression of dementia correlates with indices of immune activation as well as levels of iNOS and gp41 [358]. Moreover, it has been demonstrated that the viral protein Tat induces microglia activation and, consequently, iNOS expression both *in vivo* and *in vitro* [232, 359].

4. NITRIC OXIDE AND NEUROPROTECTION

In addition to its action as a neurotoxin and short-living physiological messenger, NO has also been described as a neuroprotectant.

4.1 Control of Redox State and Scavenging Action

NO-mediated neuroprotective reactions seem to utilize a relatively common biological trigger, i.e. S-nitrosylation or nitration of proteins, a mechanism that accounts for the regulation of many normal and abnormal biological functions. These types of protein modification are particularly attractive as a regulatory mechanism since they can be reverted depending upon the redox state of the cell [140]. Redox modulation by covalent modification of thiol groups on protein residues can regulate protein functions directing them towards neuroprotection. Recent work has suggested that S-nitrosylation of critical cysteine thiols of the NMDA receptor [360], of p21ras during MAP kinase signalling [129], and of the active site of the caspase enzymes [263], can decrease the activity of these proteins, thereby contributing to neuroprotection. Thus, depending on its redox state, NO can contribute to neurotoxicity, via formation of peroxynitrite and superoxide anion, or provide neuroprotection by down-regulating the activity of both the NMDA receptor and its downstream activation of p21ras and caspases. The mechanisms of the protective effect of NO related to inhibition of the NMDA receptor, however, remain controversial. One hypothesis is that NO nitrosylates thiol residues of the redox modulatory site leading to the formation of disulfide bonds [361]. These authors proposed that free sulfhydryl groups on the NMDA receptor-channel

complex react to form one or more S-nitrosothiols in the presence of NO. If nearby thiol groups react in this manner, they can form a disulfide bond, which is thought to constitute the redox modulatory site of the receptor [360]. These reactions with NO can afford protection from NMDA receptor-mediated excitotoxicity. Another hypothesis is that NO, or a derived species, perhaps a NO-metal complex, exerts an allosteric action on the NMDA-receptor protein that facilitates the blockade of the receptor by divalent ions [362]. These authors reported that in cerebellar granule cells, NO decreases the NMDA channel conductance and opening probability, in voltage-dependent and -independent manners, respectively, by acting on an extracellular site different from the redox, glycine, and pH modulatory sites. In fact, removal of trace concentrations of metal ions in the external medium by means of metal ion-chelators, significantly reduced the inhibitory action of NO on NMDA currents indicating that divalent ions are required for the blockade of NMDA receptors by NO donors. It has also been demonstrated [363] that endogenous production of NO can decrease NMDA receptor activity, indicating the potential physiological importance of this effect. In cultured neurons, NO was shown to reduce Ca^{2+} influx and Zn^{2+} binding through voltage-gated Ca^{2+} channels and so to reduce Zn^{2+} neurotoxicity [364]. Zn^{2+} neurotoxicity was attenuated when NOS activity in the cultures was induced by exposure to cytokines, exogenous NO was added or its production was pharmacologically enhanced. The authors proposed that NO could attenuate Zn^{2+} neurotoxicity by reducing its entry through Ca^{2+} channels, by reacting with key thiol groups. Another example of beneficial S-nytrosylation reactions that can prevent neuronal death, involves caspases. NO can block apoptosis by either inhibiting active caspase or their downstream actions [263, 365, 366]. Activation of caspases can be prevented by cGMP-dependent mechanisms, by nitrosylation of upstream signalling systems or, as recently shown, by inhibition of mitochondrial complex [291].

Oxygen/nitrogen radical-mediated oxidation is a major mechanism of post-traumatic neurodegeneration. Immediately following spinal cord injury, an oxidative process begins with the rise of superoxide anions, hydrogen peroxide [367] and NO [368] with consequent damage to important cellular components [369]. The destructive potential of these free radicals is further enhanced by the fact that endogenous antioxidants only increase at later stages after spinal cord injury. NO, produced by NO donors, or the NO precursor (L-arginine) have been shown to be neuroprotective in these lesions by improving blood flow to injured neural tissue and, thereby, reducing the risk of ischemia [370, 371, 372] and by diminishing excitotoxicity through nytrosilation of NMDA receptors [360]. As discussed before, NO, under certain conditions, can counteract the deleterious effect of reactive oxygen species: it can inhbit $ONOO^-$ oxidative chemistry through protective protein nitrosation; it can scavenge superoxide anions minimizing the deleterious effects of the Fenton reaction; it can react with alkoxyl and peroxyl radicals, thereby inhibiting radical-chain propagation reactions. NO has also been reported to ameliorate the neurotoxicity produced by H_2O_2 [373]. When primary cultures of rat mesencephalic dopaminergic cells were exposed to hydrogen peroxide or to hypoxanthine/xanthine oxidase, there was a dose-dependent degradation of the dopamine uptake and release mechanism. However, in the presence of NO released from NO donor, the cytotoxicity resulting from superoxide or hydrogen peroxide was markedly abrogated [373]. It was also demonstrated that exogenous NO can protect nigral neurons from oxidative injury by scavenging hydroxyl radicals [374] and protect against MPTP-induced, hydroxyl radical-mediated nigrostriatal lesions, acting as an antioxidant [375]. Kanner *et al.* showed that NO could prevent the destruction of hemoproteins by hydrogen peroxide and abate Fenton-type reactions [286]. As stressed before, one of the primary targets of oxidative stress is DNA. When treatments of supercoiled DNA plasmid were done with ferrous iron/hydrogen peroxide in the presence of a NO donor, complete protection was observed [376]. This clearly suggests that the presence of NO can abate the Fenton-type oxidative damage to DNA. Moreover, several studies have suggested that NO can act as a chain breaking antioxidant in lipid peroxidation reactions and play a beneficial role in diseases such as atherosclerosis [377, 378, 379]. Finally, neuroprotective effects of NO have been demonstrated in brain models *in vivo* [127]. Oxidative stress increases NO and glutathione (GS) while depletes reduced glutathione (GSH), resulting in the generation of a more potent antioxidant S-nytroglutathione (GSNO) and providing additional neuro-protection. Collectively these data demonstrate that NO can protect cells from the deleterious effects of peroxides and the resulting formation of toxic ROS.

4.2 Nitric Oxide in Survival and Neuroprotection

To understand the ways by which NO achieves its multiple effects and eventually to use it for therapeutic action, studies have focused on characterization of the enzymes responsible for NO production, identification of its target molecules and detection of the mechanisms by which NO alters these targets. Cell types shown to be protected from apoptosis by NO include lymphocytes [380, 381], endothelial cells [365], eosinophils [382], multiple cell lines [383, 384], hepatocytes [385], and certain neurons [386, 387]. Ample evidence suggests that the formation of NO excess by the stimulation of the inducible isoform of NOS (iNOS) or exogenous treatment with a high concentration of NO donors are responsible for the cytotoxicity against several type of cells [388], including macrophages [389], astrocytes [390], differentiated PC12 cells [162, 165] and primary brain cultures [229]. Under these cytotoxic circumstances, NO is considered to be a mediator of apoptotic or necrotic cell death. The controversial action of NO cannot be explained solely on the basis of its different chemical forms and reactivity. Often the cell type, as well as its state of differentiation, is a relevant determinant of the survival- or death-promoting effect of NO. Exposure of neuronal PC12 cells differentiated by nerve growth factor, to TNF-α and bacterial lipopolysaccharide (LPS) resulted in de novo synthesis of iNOS leading to cell apoptosis [165]. Furthermore, exposure of undifferentiated PC12 cells to low concentrations of NO donors resulted in dose- and time-dependent death [391]. Contrasting evidence, however, suggested that NO has neurotrophic and neuroprotective effects on serum-deprived PC12 cells [386], cerebellar

granule cells [136,392, 393] and neuroblastoma cell cultures [394, 395]. A novel pathway accounting for NO survival effect, has recently been disclosed using primary cultures of cerebellar granule cells [136]. We have, indeed, demonstrated that chronic inhibition of NO production by differentiated granule neurons resulted in their progressive apoptotic death, accompanied by downregulation of a pathway involving the survival kinase Akt. Moreover, in embryonic motor neurons NOS inhibition resulted in apoptosis of motor neurons cultured in the presence of brain-derived neurotrophic factor (BDNF), an effect blocked by excess L-arginine [324, 387]. In dissociated cultures of dorsal root ganglia, the application of blockers of NOS caused selectively death of those neurons expressing NOS [396]

Multiple mechanisms or pathways have been identified by which NO may inhibit apoptosis. Given that activation of soluble guanylate cyclase occurs almost universally in response to NO, cGMP has received primary consideration as the mediator of NO actions. In the brain, the structures capable of synthesizing cyclic GMP in response to nitric oxide were compared with the anatomical localization of NOS containing neurons [397]. NO-responsive soluble guanylate cyclase and NOS were usually juxtaposed at very short distances in the rat brain, strongly supporting the proposed role of NO as an endogenous activator of the soluble guanylate cyclase in the central nervous system. NO protection against apoptosis is dependent on cGMP in spinal cord motoneurons [387] and in cerebellar granule cells [136, 392, 393]. Inhibition of NO synthase by L-NAME or of soluble guanylate cyclase by ODQ, resulted in apoptosis of cerebellar granule cells, which was reverted by addition of a cell permeable analog of cGMP [136]. Moreover, in experimental conditions in which ethanol exposure induced cell death in the same cultures, NMDA enhanced survival of these neurons by a mechanism that involved the NO-cGMP pathway [392]. Other researches have demonstrated a similar cGMP/PKG-mediated survival action of NO on beta-amyloid-mediated cell death in PC12 cells [398], serum-deprived PC12 cells [399], neurons of the nigrostriatal dopaminergic system [222], and human SH-SY5Y neuroblastoma cells [400].

A primary action of elevated cGMP levels is the stimulation of PKG, the major intracellular receptor protein for cGMP, which phosphorylates various substrate proteins. The effect of cGMP on apoptosis may be mediated through PKG [385], but how PKG achieves this effect is not known. One possibility is that PKG brings to Akt activation through phosphorylation of upstream protein(s) [136, 401]. In rats, dorsal root ganglia neurons are dependent on NGF for survival [396]. In dissociated cultures, it was demonstrated that the increase in nNOS expression is regulated by nerve growth factor and that inhibition of NO production or cGMP synthesis resulted in preferential apoptosis of neurons expressing nNOS [396].

Some evidence has recently been obtained that nitric oxide signaling may be functionally coupled with the activation of the transcription factor, CREB, in nerve cells. A recent paper [402] has demonstrated that PKG activation by NO, contributes to the increased phosphorylation of CREB occurring during the late phase of hippocampal LTP. This NO-dependent pathway seems also involved in the induction of another well known form of synaptic plasticity, the long-term sensitisation in sensory neurons of *Aplysia* [403]. In other cellular systems, NO-induced CREB phosphorylation through guanylate cyclase/PKG pathway has also been demonstrated [404, 405]. Evidence for a similar functional link has also been obtained *in vivo*, where light stimulation during the dark phase is able to elicit in the hypothalamus, a NO-dependent CREB phosphorylation, which contributes to reset circadian rithms [406]. The previously reviewed data suggest that CREB activation is one of the ways by which NO exerts its action on neuronal survival and synaptic plasticity. We recently reported that NO shortage downregulates CREB phosphorylation in cerebellar granule neurons, decreases the expression of the CREB-regulated antiapoptotic gene, Bcl-2, and that similar effects are obtained by interfering at different levels with the main cellular pathway linking NO to CREB, namely the one related to guanylate cyclase and PKG activation [393]. This demonstration that CREB activity may be regulated by NO, in addition to classical neurotrophins, is critical to achieve better understanding of NO function in the brain. The human neuroblastoma cell line SK-N-BE, that represents a good model to study the effect of NO activity on neuronal survival and differentiation, was used to show that spontaneous apoptosis of SK-N-BE cells overexpressing the p75 neurotrophin receptor could be rescued by either high endogenous NOS activity or presence of NO donors in the culture medium [394]. We recently demonstrated that overexpression of nNOS isoform protects these neuroblastoma cells from death induced by trophic factor withdrawal and that this protective action occurs in parallel with enhanced CREB activity [395]. Taken together, these results support the notion that NO acts as a survival-promoting agent of neural cell through CREB activations.

A sustained NO release may produce multiple and complex biological responses mediated not only through the activation of the NO-cGMP pathway, but also through direct modification of proteins, DNA and other cellular components by NO itself. As noticed before, NO may inhibit caspase-3-like protease activity through nytrosilation. It is now known that caspase-3-like protease activation can promote Bcl-2 cleavage and mitochondrial cytochrome c release and that these events can lead to further downstream caspase activation. It was recently shown [407] that NO suppresses a key step in the positive feedback amplification of apoptotic signaling by preventing Bcl-2 cleavage and cytochrome c release. NO can directly inhibit caspase activity through *S*-nitrosylation of the active cysteine conserved in all caspases [365, 401]. However, it is not clear whether NO interferes only with activated caspases or whether NO blocks pro-caspase processing and activation. The anti-apoptotic protein Bcl-2 is increased by cytokine-induced iNOS or NO donors in endothelial cells [408]. Recent results pointed at Bcl-2 as one gene negatively regulated in cerebellar granule cells, at both mRNA and protein levels, by L-NAME and ODQ treatments and rescued by a NO donor [393]. These results point at Bcl-2 as an anti-apoptotic gene whose expression, under the transcriptional control of CREB, is regulated, together with other previously determined intracellular pathway [409], also by the NO-cGMP cascade. In SH-SY5Y neuroblastoma cells non-lethal serum deprivation-stress enhances the tolerance to a subsequent

lethal oxidative stress and also to 1-methyl-4-phenyl-pyridinium (MPP(+) neurotoxic challenge, in parallel with increased expression of nNOS, and then NO production [400]. In this system, the NO-cGMP-PKG pathway mediates the preconditioning-induced upregulation of antiapoptotic protein Bcl-2 and the downregulation of adaptor protein p66(shc), suggesting that NO-PKG-Bcl-2 signaling pathway plays an important role in the preconditioning-induced neuroprotection against oxidative stress. Some of the factors associated with apoptosis that are activated when nNOS activity is blocked in dorsal root ganglion neurons have been studied [410]. Marked elevation of bax was observed within a few hours of NOS inhibition in nNOS containing neurons, whereas pre-treatment of cultures with L-arginine or cGMP completely abolished this effect. These results suggests that NO has a neuroprotective action by inhibiting bax, and caspase-3 and -9 activation. NO has also been shown to be implicated as a mediator of Ras activation by NMDA receptor stimulation [259]. During oxygen-glucose deprivation preconditioning in neurons, a signaling cascade is initiated by activation of NMDA receptors, Ca^{2+} influx, and production of NO, which leads to the development of neuronal tolerance to the toxic insult in a Ras/Erk-dependent manner [411].

NO has also been shown to affect gene expression at the level of transcription [412, 413, 414] and translation [407, 415]. Haby and co-workers [413] showed that NO promotes immediate early genes such as c-fos, c-jun, junB, and junD activation and AP1 binding enhancement through the stimulation of the NO-cGMP pathway. Besides these genes, NO-related activity from cytockine-induced iNOS or the NO donor SNAP induced the expression of inducible heat shock protein 70 (HSP70) in rat hepatocytes [385]. Antisense oligonucloetide to HSP70 blocked its induction by SNAP, and abolished SNAP protection from TNF-α [385]. A short visual summary of some of the cellular pathways leading to NO-dependent survival and neuroprotection, is given by ("Fig. (**5**)").

4.3 Controversies on Neurotoxicity/Neuroprotection from *In Vivo* Studies

Despite recent therapeutic advances, there is still no effective treatment to prevent secondary autodestructive processes that occur after a traumatic injury to the central nervous system, such as glutamate excitotoxicity, calcium overload, oxidative stress, and ischemia [416]. Among the various drugs that have been suggested to have a neuroprotective effect, NO is, at first glance, an unlikely candidate. NO is, indeed, best known for its neurotoxicity in the oxidized environment of the injured neurons that for its potential as neuroprotectant of damaged neurons. Controversial results on the NO action stem from the literature when experiments of neurotoxicity/neuroprotection are performed, and in particular when they are carried out *in vivo*. Nervous system damage in response to an insult may lead to acute or delayed neuronal death. Glutamate has emerged as an important mediator of ischemic brain injury [417]. In general, NMDA receptor and non-NMDA receptor-mediated excitotoxic injury results in neurodegeneration along an apoptosis-necrosis continuum. The effects of

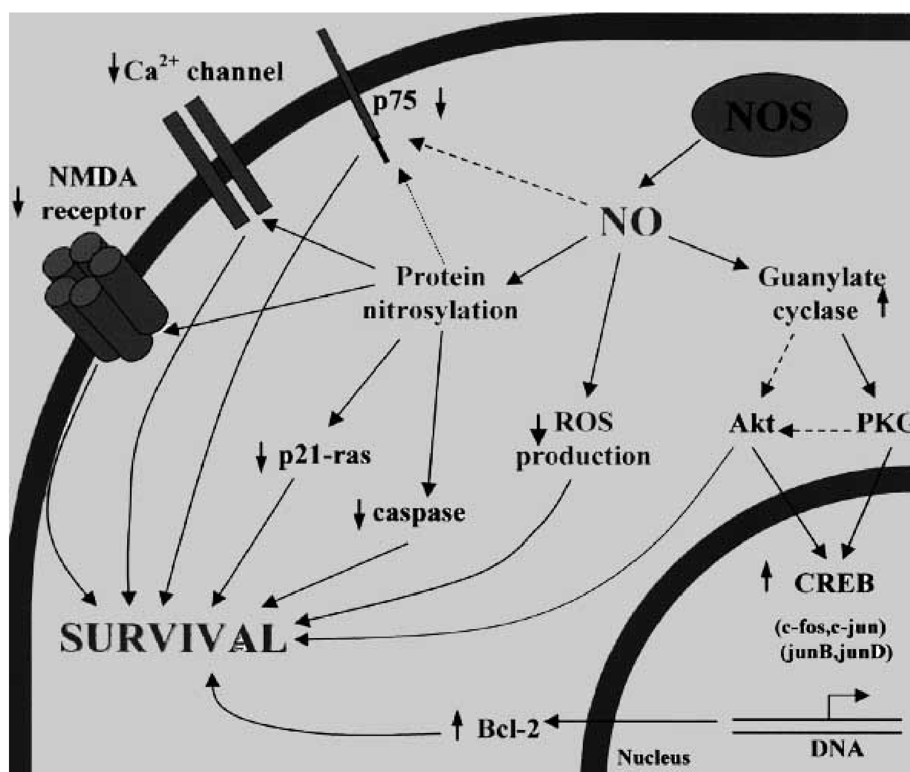


Fig. (5). Composite drawing summarizing some cellular signaling pathways mediating physiological and neuroprotective effects of NO.

neuronal injury depend on factors including the degree of brain maturity and the site of the lesion. The landmark observation that activation of NMDA receptors generates nitric oxide (NO) in a Ca^{2+}-dependent manner [128], raised the possibility that NO participates in glutamate neurotoxicity. This hypothesis gained momentum from the demonstration that inhibition of NO synthesis attenuated NMDA neurotoxicity in neuronal cultures [179] and reduced the brain damage produced by occlusion of the middle cerebral artery in mice [418]. NO was also found to be neurotoxic in models of focal cerebral ischemia [418, 419, 420], in hippocampal slice studies [421] and in excitotoxicity experiments involving intrastriatal infusion of NMDA [422]. 7-nitroidazole, a relatively specific inhibitor of the neuronal isoform of NOS *in vivo*, is efficacious against focal ischemic lesions [423], it blocks MPTP neurotoxicity *in vivo* [422] and ameliorates striatal lesions produced by NMDA, kainic acid or AMPA [424]. It was further demonstrated that nNOS deficient mutant mice are resistant to MPTP, suggesting that neuronally derived NO might be involved in the neurotoxic action [205]. However, NO protects dopaminergic neurons and cultured rat astrocytes from MPP+ induced toxicity [425, 426] and erythropoietin is protective in (MPTP)-induced mouse model of Parkinson's disease via increasing NO production [427].

NOS inhibition was found to either augment, diminish or have no effect on neurotoxicity following the infusion of excitotoxins [428, 429, 430, 431, 432]. In general, experiments aimed at obtaining neuroprotection through inhibition of NO production tend to underscore the fact that long-lasting shortage of NO may be detrimental when neuroprotective mechanisms related to its physiological action are severely impaired. This possibility was substantiated by a recent work from our laboratory [432]. We showed that the damage caused by intrastriatal injection of ibotenic or kainic acid was aggravated in rats subjected to chronic NOS inhibition. These results suggest that, contrary to what has often been reported for short-term, mild inhibition of NO production, chronic and sustained NOS inhibition may exacerbate neuropathology [432]. Moreover, recent *in vivo* results indicate that nitric oxide and S-nitrosothiols can protect brain dopamine neurons against oxidative stress. Scavenging hydroxyl radicals through NO donors was protective against ferrous citrate induced neurotoxicity in rat nigrostriatal dopaminergic system [433, 434] and an NO releaser, nytroglicerine, attenuated the MPP+-induced dopamine depletion in rats [375].

As a neurotoxin, NO may mediate the ischemic excitotoxic brain injury induced by glutamate release and NMDA receptor overactivation. On the other hand, as a signaling molecule, NO may induce an increase in blood perfusion of ischemic penumbra in the early stages of cerebral ischemia [435]. Experimental interventions aimed at increasing endogenous and exogenous NO or supplying excess NOS substrate, L-Arg, gave contrasting results on infarct size in cases of focal cerebral ischemia [436, 437, 438, 439, 440]. The use of relatively selective inhibitors of NOS isoforms has only partly clarified the role of NO in focal cerebral ischemia. Selective inhibitors of nNOS consistently reduced infarct size and attenuated functional impairment [441, 442, 443, 444]. Moreover, delayed treatment with aminoguanidine, a selective inhibitor of iNOS, reduced infarct size after both transient [445, 446] and permanent [447, 448] focal ischemia, and enhanced neurological recovery [448]. In contrast, the NO donor sodium nitroprusside reduced the infarct size after focal cerebral ischemia in rat [440] and tolerance against ischemic neuronal injury was induced by volatile anesthetics, a process in which iNOS seems to be critically involved [449]. Experiments with mutant mice deficient in selected NOS isoforms have complemented the results obtained with selective NOS inhibitors. Infarct size was reduced in mice lacking nNOS [189, 450] or iNOS [451], while increased brain damage was produced in eNOS knockout mice [452]. This suggested that NO coming from different sources (neurons, brain vessels, glia and infiltrated neutrophils) could differently influence the evolution of brain damage at different times after an ischemic insult [191]. NO-enhancing reagents have been shown to lead to functional improvement in models of cerebral ischemia [371, 372], ischemic retina [453], as well as in cortical trauma [370]. Preconditioning adaptation induced by transient ischemia can also increase brain tolerance to oxidative stress, but the underlying neuroprotective mechanisms are not fully understood. It has been demonstrated that a non-lethal serum deprivation-stress for 2 h (preconditioning stress) that increased the expression of nNOS RNA and then NO production, enhanced the tolerance to a subsequent lethal oxidative stress (24 h serum deprivation) and also to MPP+ [400, 454]. The evidence presented above is a short summary of the extensive literature on the issue and clearly indicates the dual role played by NO in the mechanisms of neuroprotection/neurodegeneration. Some of the reviewed data suggest that the effect of NO on damaged brain depends on the stage of evolution of the degenerative process and on the cell type producing NO. Therefore, modulation of NO production in the damaged brain could be a useful, but not easily attainable, therapeutical goal for neuroprotective strategies.

5. PHARMACOLOGICAL APPROACHES FOR THE MANIPULATION OF BRAIN NITRIC OXIDE: ADVANTAGES AND PITFALLS

To devise therapeutical tools able to interfere with the various steps of NO production, biological activity, decay and/or inactivation poses, at first glance, almost unsurmountable problems. The first, and the most important, of these problems clearly emerges from the data summarized in the previous sections and is related to the Janus-faced properties of the molecule. Any pharmacological treatment aimed to decrease NO availability has to be confronted with the goal of avoiding its excessive production without too severely hampering the essential physiological mechanisms carried on by the molecule. On the other hand, drugs targeted to increase NO availability and/or to reinforce or prolonge the NO-dependent biochemical cascades inside the cell should be balanced in their action, so that the risk of uncontrolled bursts of NO production is avoided. As outlined in the previous sections, this double-faced nature of NO is reflected in almost all of its biological actions, from nitrosylation that can favourably or unfavourably change the function of a target protein to the

pro-oxidant or the free radical scavenging function exerted depending on the particular cellular milieu. A second problem is related to the chemical nature of NO, a rather "unconventional" molecule when compared to other bodily chemical messengers: a very small, short-lived gaseous molecule, freely diffusible through cell membranes and intercellular spaces, synthetized in bursts depending on the physiological demand and easily reacting with many biological components. These erratic chemical features, make NO a difficult objective for any pharmacological intervention aimed at modifying its action in time and space, i.e. to target it in specific cells and at specific physiological steps. For this reason, the most detailed knowledge of the NO-dependent biochemical cascades may be of the utmost importance in order to devise strategies of pharmacological intervention able to interfere with downstream events elicited by NO activity.

5.1 Nitric Oxide Synthase Inhibitors

Focusing primarily on the neurodestructive potential of excessive NO production [455] and on hypotheses viewing uncontrolled NO availability as a pivotal factor in brain aging [182, 456], NOS inhibitors have been proposed as potentially useful in human therapy [422, 457]. As outlined in the previous sections, these data appear highly controversial, as various groups have reported protection or aggravation of neurodegenerative lesions by inhibiting NO production from different sources through the use of general NOS inhibitors or of inhibitors claimed to be somewhat specific for different enzymatic isoforms. A reassessment of the validity of this approach, taking into account both theoretical and practical issues, may thus be timely and helpful for future research and perspective therapeutical strategies. The issue of selectivity of action towards different NOS isoforms is particularly critical as, ideally, silencing one NO source by leaving the others undisturbed could represent the best way to understand NO physiopathology in order to design therapeutically useful treatments. In the first section of the present article the structural and functional complexity of NOS isoforms has been outlined, together with similarities and differences in mechanisms of catalysis and regulation. Theoretically, inhibition of NO production may be achieved by means of various pharmacological interventions, from preventing L-arginine entry and accumulation inside the cell to interfering with the availability of cofactors essential for enzymatic activity or inhibiting electron flow through the NADPH/flavins pathway [65, 458, 459, 460]. Practically, however, drugs targeted to the binding of the substrate to the catalytic site of the NOS molecules have been prevailingly devised and tested. On one hand, this approach has the advantage of interfering in a very direct way with the enzyme job, leaving untouched general regulatory mechanisms of vital importance for cell function. On the other hand, an obvious disadvantage may be represented by the fact that such an approach possesses, in principle, a limited potentiality to achieve effective inhibition of the different NOS isoforms in a selective way. However, sequence homology among the different NOS isoforms is not much higher than 50% [65, 461] and, while the active catalytic site is relatively well conserved, nearby regions are much more variable [2, 39]. This leaves reasonably good chances of finding inhibitors endowed with relatively high selectivity towards the different isoforms by designing molecules able to interact with the active site and, at the same time, with the adjoining sites most variable among isoforms. Several L-arginine analogs have been tested in order to provide competitive inhibition of NOS by binding to the catalytic site, with the possible additional advantage of decreasing the availability of the natural substrate by also competing with the cell transport mechanisms. N^G-monomethyl-L-arginine (L-NMMA), L-N^G nitroargine (L-NA) and its methyl ester (L-NAME), much more water-soluble and requiring cleavage by endogenous esterases to give the active compound, have been used [65, 462, 463, 464]. These analogs provide a significant degree of NOS inhibition but show little selectivity towards enzyme isoforms [65]. Furthermore, the actual extent of inhibition, and in particular the time window of it, appears somewhat variable depending on the dosage used and the treatment regimen adopted. By using a sub-chronic treatment of at least 8 days of duration and based on administration of 60 mg/Kg/day L-NAME divided in two daily doses, we obtained a standing inhibition higher than 95% of calcium-dependent NOS catalytic activity both in neonatal and adult rats [465]. This degree of inhibition appears close to the maximum that can be obtained through pharmacological NOS inhibition and is not very far from the condition of mice knockout for single NOS isoform [131]. Among arginine derivatives, N^{ω}-allyl-L-arginine and N^{ω}-propyl-L-arginine appear to affect catalytic activity with a mechanism more complex than simple competitive inhibition and display relative selectivity towards different isoforms by inhibiting some of them at concentrations much lower than others [466, 467, 468]. Other amino acid analogs are effective NOS inhibitors, sometimes showing some degree of selectivity, as it is the case for S-methyl and S-ethyl derivatives of L-thiocitrulline whose action is exerted through block of the *heme*-dependent oxygen activation [469, 470]. Derivatives of other amino acids, such as N^5-(1-iminoethyl)-L-ornithine (L-NIO) and N^6-(1-iminoethyl)-L-lysine (L-NIL) are effective NOS inhibitors showing a limited degree of selectivity [460]. However, several analogs of these compounds were pharmacologically very interesting as they displayed noticeable differences in their potency as inhibitors of different NOS isoforms [468].

A second class of NOS inhibitors comprises compounds not derived from amino acids and of varying chemical nature. One of the first compounds to be tested was aminoguanidine, an agent effective in animal models of inflammatory diseases and, therefore, supposed to be highly selective for iNOS [471, 472, 473]. However, at the concentrations reported to be effective *in vivo*, also constitutive NOS isoform are likely in part inhibited and, furthermore, the compound additionally inhibits other iron- and copper-containing enzymes such as catalase [474]. Isothiourea derivatives are very potent but poorly selective inhibitors of NOS isoforms, even if some degree of selectivity is obtained by introducing small side chain substitutions [475, 476]. Some heterocyclic compounds of the group of nitroindazole and imidazole such as 7-nitroindazole (7-NI) and its 3-bromo derivative or trimethylphenylfluoro-imidazole (TRIM) are potent inhibitors of all NOS isoforms *in vitro* [477], through

binding at the *heme* site but also interfering with the arginine and tetrahydrobiopterin binding sites [478]. *In vivo*, however, 7-NI has been reported to be a relatively specific inhibitor of nNOS, which may result from differential compartmentalization in the brain and from preferential uptake from cells expressing this isoform. This conclusion relies on multiple evidence including anti-nociceptive activity, anti-convulsive potency, decrease of cerebral blood flow [479, 480, 481] and neuroprotection in experimental models of neurodegeneration as mentioned above and further discussed below. A major problem with 7-NI, that is also likely at the basis of the variability of the reported results, is related to the potency and the time course of the inhibition obtained. After i.p. administration of 30 mg/Kg in the rat, maximal inhibition was recorded after 30 minutes [481]. The same study, however, pointed at the transitory and incomplete features of 7-NI inhibition, by demonstrating that the best inhibition obtainable after multiple i.p. injections (30 mg/Kg every 4 hours for 20 hours) was around 60% four hours after the last administration. In a subsequent investigation, doses giving the maximum effect (40-80 mg/Kg), resulted in 55-85% inhibition of the calcium-dependent catalytic activity 30 minutes post injection in various brain regions of the rat [483]. Also in this case, the inhibitory effect was short-lasting, as the catalytic activity had substantially recovered four hours later, particularly in forebrain regions [483]. In agreement with these results, we only measured a minor inhibition (~30%) after daily administration of 60 mg/Kg 7-NI for 8 days, 8 hours after the last injection [432].

Tris-malonyl-C(60)-fullerene derivatives, have recently been demonstrated to inhibit the various NOS isoforms with IC(50) values similar for nNOS and eNOS, but about fivefold higher for iNOS, by interfering with electron transfer between dimer subunits [484]. An interesting attempt to combine NOS inhibition with lipid peroxidation scavenging was recently performed by combining in the same molecule a "NOS pharmacophore" with a vitamin E analog (compound 80933) [485]. This compound, that exhibited a remarkable specificity towards nNOS inhibition, substantially reduced lesion size in animal models of head trauma and cerebral ischemia when adminstered prior to lesion, and was also beneficial towards neurological outcome when administered up to 8 hours after ischemia onset [485]. The anti-estrogen drug tamoxifen, widely used in clinical trials for breast cancer treatment, was found to be a potent and selective inhibitor of constitutive NOS by antagonizing calmodulin activation of the catalytic activity [486]. To complete the picture of NOS inhibitors, it should be mentioned that endogenous NOS inhibitors may exist and be considered for future use as pharmacological tools. Such an endogenous inhibitor has been identified in the rat brain in the form of an 82-amino acid protein inhibiting nNOS (PIN) [111, 487].

As in part outlined in the previous sections, studies based on nonselective or presumably selective inhibitors of NOS gave rise to highly controversial results when concerned with *in vivo* models of cerebral ischemia, excitotoxicity and oxidative stress-related neurodegeneration resulting from mitochondrial energy failure [205, 206, 375,

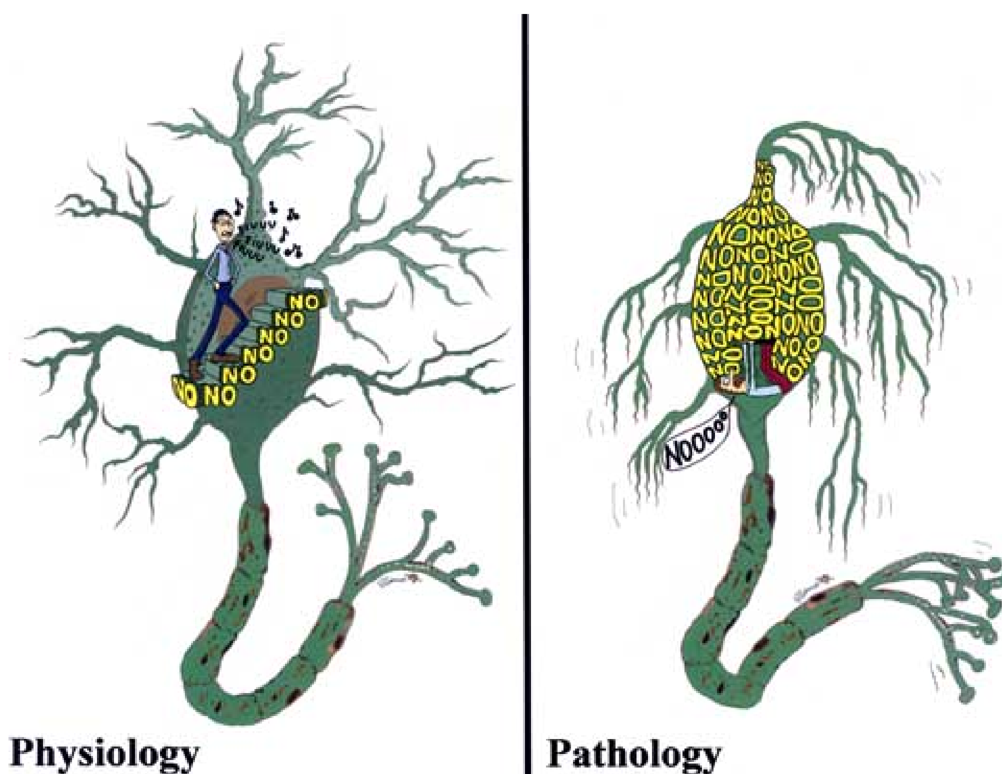


Fig. (6). Cartoon-like representation of the dual role of NO in Physiology and Pathology.

419, 422, 432, 488-495]. These contradictory observations must be confronted with results stemming from mice knockout for different NOS isoforms. Mice knockout for nNOS are less sensitive than wild-type ones to ischemic brain damage and MPTP dopaminergic neurotoxicity *in vivo*, and their neurons put in culture show reduced sensitivity to glutamate excitotoxicity [189, 205, 450, 496]. On the other hand, ischemic damage is exacerbated in mice lacking the eNOS isoform and this aggravation can be attenuated by the unselective NOS inhibitor, L-NA [452]. Putting together the results from isoform-selective knockout mice with the fact that neuroprotection is better granted by inhibitors showing some selectivity for nNOS, while unselective inhibitors often aggravate the damage, one is tempted to agree with explanations attributing a prevailingly neurodegenerative role to nNOS and a prevailingly neuroprotective role to eNOS through a beneficial vascular effect [65, 435]. In this framework, iNOS will play a delayed neurodegenerative effect when its activity is induced in glial cells brought to an activated state by neuronal degeneration [65, 435]. These conclusions, while supported by a large, but not unanimous, consensus may result too oversimplified and not correctly reflecting the complexity of the physiological functions of NO in the brain, as well as the possible pitfalls of different ways to pharmacologically manipulate its levels and site of action. In view of the mounting evidence supporting an essential role of NO in different steps of neural development, from neurogenesis to survival, differentiation and synaptogenesis [136, 162, 322, 393, 395, 497, 498, 499], the fact that no patent alterations in brain building are evident in single- and double-isoform knockout mice, clearly suggests the existence of relevant compensatory mechanisms occurring during development and likely extending their influence throughout adult life. This may confer to the knockout mice functional properties that distinguish them from wild-type animals but not necessarily reflect a direct effect of the genetic deletion. To exemplify this issue, one may discuss a result obtained in nNOS and eNOS knockout mice [500]. Microdialysis experiments were performed in different brain regions of mice, providing neuronal activation through conventional depolarization techniques (high potassium in the perfusion medium) or by specifically targeting NMDA receptor, and measuring variations in the release of glutamate and GABA. While no differences were measured after high potassium depolarization, NMDA receptor stimulation increased glutamate release in the cortex, but not in the striatum and hippocampus, of nNOS knockout mice while it strongly decreased GABA release in all the regions of eNOS knockout mice. The authors took these results as an indication that the two enzyme isoforms had different roles in the modulation of excitatory/inhibitory neurotransmission, likely as a consequence of different neural localization [500]. This interpretation relies on the original concept that NO functions primarily as a retrograde synaptic messenger, only stimulating neurotransmitter release from a restricted number of adjoining synapses, ideally only from terminals pre-synaptic to the coupled post-synaptic neuron originating the NO efflux. This view is no longer tenable in the light of estimate of the actual diffusion of the molecule in brain tissue [reviewed in 131]. It is admitted, indeed, that NO produced by a single cell can diffuse and be effective in a volume of tissue enclosing thousands of neurons and millions of synapses [131]. It seems unlikely that NO produced in the same brain region, for example in the cortex, has differential effects depending on its origin from different NOS isoforms localized in neuronal sub-populations or other cellular elements whose reciprocal distance is smaller as compared to the diffusion volume of NO. In conclusion, the differential sensitivity to ischemic damage of nNOS and eNOS knockout mice may well be related to an excitotoxic increase in glutamate release in nNOS knockouts and to a neuroprotective increase in GABA release in eNOS knockout, but both of them may not be necessarily related to the specific genetic defect.

Results arising from the use of NOS inhibitors, may favor the idea that selective inhibition of nNOS is neuroprotective, while unspecific inhibition of multiple NOS isoforms, including eNOS, is an aggravating factor in neuropathology. Evidence supporting this conclusion is not univocal and mainly resides in the fact that nNOS specific inhibitors, such as 7-NI, often result in neuroprotection. Following this line of reasoning, one would expect to find no beneficial effect, or an aggravating effect, by using unselective inhibitors such as L-NA and L-NAME, which is not exactly the case as in some experimental models also these inhibitors are neuroprotective. Also in this case, there are reasons to believe that to attribute "bad" or "good" properties to nNOS and eNOS respectively, may oversimplify a more complicated issue. As outlined above, 7-NI is a relatively weak and short-lasting inhibitor, while appropriate schedules of treatment with unselective inhibitors like L-NAME may ensure strong and long-lasting inhibition of NO formation. This suggests that, while the goal of finding inhibitors more and more specific for different NOS isoforms remains central, much more attention should be given to the characterization of the extent and the time span of the inhibition achieved with different treatment schedules. It is unfortunate, that in many of the reported researches investigators have omitted to check the extent and the duration of the inhibition of NO production achieved with the specific treatment adopted, and to correlate it with the neuroprotective or neurodegenerative results obtained. It is not unlikely that in many of these researches, similarly to what is suggested by studies in which this essential control was performed, neuroprotective effects were obtained when inhibition of NO production was only mild and transient, while an aggravating action was associated with conditions of strong and long-lasting inhibition. Indeed, many of the reported researches converge to indicate that the extent of the inhibition of NO production is even more critical than the specific source of production blocked in order to determine the final result of the pharmacological treatment. If this is true, it seems mandatory that future researches, in addition of pursuing the road towards specific inhibitors of NOS isoforms, give much more attention to pharmacodynamics of the perspectives drugs.

5.2 Nitric Oxide Donors

Due to the many essential functions played by NO in brain physiology and to the neuroprotective action ascribed to this molecule under certain neurodegenerative circumstances, it is not surprising that the search for drugs able to increase NO availability has been pursued at least as

much as the search for drugs able to block its production. Among these drugs, the search for NO releasers suitable for supplementing cells with NO in a way somewhat similar to the physiological one, has been more consistently exploited. In this picture, it is worth to remember that the oldest therapeutical NO donors are nitroglycerin and related substances, used for medical purposes since almost a century and still in use today. These "nitrovasodilators" have been introduced in medical practice as agents able to help patients in cases of angina pectoris [439, 501]. While in principle relatively simple, the actual action and pharmacokinetics of NO releasing drugs may be rather complicated. As outlined in recent reviews [439, 502], NO from different donors can be produced non-enzymatically or through an enzymatic reaction, it may require oxidation, reduction or interaction with thiols to become active. Furthermore, different compounds may result differentially sensitive to pH, light, temperature, oxygen pressure and so on. In several cases, NO donors do not directly release nitrogen monoxide but redox forms of it (NO^+, NO^-, $NO^{\cdot\bullet}$) whose rate of conversion into NO can be profoundly influenced by their reactivity towards biomolecules differentially present in the biological environment. In addition to all these problems, *in vivo* administration poses further limitations related to the difficulty of correctly estimating actual NO concentration over time due to the high reactivity of the molecule as well as to unwanted systemic effects. The best example of undesirable side effects is vasodilation with consequent generalized decrease of systemic blood pressure and variability in local hemodynamics in different body districts. Concerning compounds intended to act at the brain level, *in vivo* administration can be further complicated by their more or less lipophylic nature and, therefore, their ability to cross the blood-brain barrier after systemic administration.

Sodium nitroprusside has been used for decades as a drug able to rapidly decrease blood pressure in hypertensive crisis. Release of NO upon *in vivo* administration requires reaction with thiols [503] or enzymatic bioactivation [504]. This is accompanied by release of cyanide [504], whose dangerous accumulation represents an important limitation for chronic or sub-chronic administration of sodium nitroprusside. A second class of compounds suited to release in a therapeutically useful way, NO, is constituted by organic nitrates, whose prototypical representative is nitroglycerin. These nitric acid esters of alchols, such as glyceryl trinitrate (GTN) or isosorbide dinitrate (ISDN) require bioactivation to release NO and this may be accomplished by either interaction with SH groups (for example from cysteine) or enzymatic catalysis through NADPH-dependent, cytochrome P450 pathway or glutathione S-transferase [501, 505, 506, 507]. S-nitrosothiols are obtained by nitrosation of primary, secondary or tertiary thiols of various organic molecules including several proteins. Representative compounds are S-nitroso-N-acetyl-DL-penicillamine (SNAP) and S-nitrosoglutathione (GSNO). In addition of acting as NO releasers upon activation [508], these compounds start complex chains of transnitrosation reactions [439], making it difficult to distinguish the effects of increased NO availability from those more directly related to nitrosation of specific proteins in target cells. Sydnonimines, such as 3-morpholine-sydnonimine (SIN-3) are a class of heterocyclic NO donors with therapeutical use in cases of attacks of angina pectoris [439].

NONOates are compounds characterized by the presence of a functional group of the general structure X-[N(O)NO]- resulting from the binding of a NO dimer to a nucleophilic residue, for example an amine, via a nitrogen atom. The rate of NO release from these compounds is relatively well predictable for a given molecule and for the given conditions of pH and temperature, and does not seem to be much affected by biological reactants encountered in *in vitro* or *in vivo* systems [439, 509]. While NO delivery from NONOates is almost instantaneous at acidic pH, the release of NO follows a much slower kinetic, even if with a reduced yield, at physiological pH. Rates of release can be evaluated from the measure of half life of the various compounds in solution. At near-physiological conditions (temperature of 37 °C and pH 7.4) half lives of different NONOates range from few minutes up to around a day (20 hours for diethylene triamine NONOate, DETA/NO; up to 30 hours for Glyco-S-nitrosothiols derived from S-nitroso-N-acetylpenicillamine (Glyco-SNAPs) [510, 511]. Furthermore, many NONOates are well-soluble in water and are relatively stable when prepared in concentrated solutions at high pH, immediately starting to release NO when diluted in physiological solutions. These properties make NONOates very attractive for experimental use, particularly in *in vitro* conditions. *In vivo*, a major limitation is represented by the fact that decomposition starts immediately after systemic administration. Therapeutical use will probably be directed towards the synthesis of prodrugs able to start NO release in the target tissues upon bioactivation [439].

A further area of possible utilization of compounds able to release NO, is in association with non steroidal anti-inflammatory drugs (NSAIDs), whose prototypical representative is aspirin. These drugs are largely used for several therapeutical purposes, first of all treatment of rheumatisms and other degenerative inflammatory diseases of joints. Modification of the molecules that make them able to act as slow NO releasers has brought to several formulations of (NO-NSAIDs), such as NO-aspirin, NO-ketoprofen, able to minimize the most dangerous side effect of NSAIDS, i.e. ulceration of gastrointestinal mucosa [502, 512]. As NSAIDs have been shown to decrease the risk and the onset of dementia of Alzheimer's type [513], NO-flurbiprofen drugs have been synthetized and they show promising results in animal models of dementia [512]. In this case, it is not unlikely that the association of an NO donor moiety is not only important to prevent gastrointestinal ulceration, but also to increase therapeutical efficacy related to the NO slow-releasing ability.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Most of the data reviewed in the present paper are highly representative of the NO paradox in brain physiopathology: a molecule essential to keep most neurons in a good status of functional activity, but a dangerous room-mate when excessively produced and accumulating in the neuron. In ("Fig. (**6**)"), a cartoon-like representation is given of this

paradoxical situation. These Janus-faced properties of NO make this molecule a very difficult target for devising drugs able to counteract its "dark side", the neuropathological consequences of uncontrolled activity, without hampering the beneficial effects of its "bright side", the many relevant functions related to neuroprotection and brain plasticity. The goal of finding drugs effective in blocking NO production in neuropathological states has been actively pursued during the last decade. This goal, however, clearly needs to be redefined in view of the mounting evidence that excessive lowering of NO levels may be very dangerous by itself and may exacerbate, instead of alleviate, ongoing neurodegenerative processes. While an increase in the degree of selectivety towards specific NOS isoforms will surely render newly developed drugs more appealing for therapeutical purposes, other aspects should be carefully considered in the design of these new drugs. Ideally, isoform-selective NOS inhibitors should ensure a not too drastic, but relatively long-lasting, inhibition of the enzyme activity in order to achieve a therapeutically useful standing decrease of NO levels avoiding, at the same time, the risk of putting NO production below the minimal level required by physiological activity. On the other hand, drugs aimed at increasing NO availability to brain in conditions of insufficient endogenous production, should accomplish this goal avoiding uncontrolled, short-lasting bursts of NO liberation and targeting in the most selective possible way the structures in which NO shortage must be counteracted. Among currently available pharmacological sources of NO release, long lasting NO donors of the class of NONOates are the closest to these ideal features. Future researches on these drugs should be able to provide therapeutical tools more selective in their targets, for example pursuing the road leading to construction of NO donors that can be preferentially taken up by the brain and activated there. Inhibitors and releasers, however, are not the only classes of NO drugs that can be hypothesized for future developments. As long as the molecular pathways mediating the cellular response to NO are being disclosed, new potential targets for pharmacological intervention become progressively available. These exciting opportunities, however, must be tempered by the fact that most of the principal signal pathways elicited by NO in target cells are not exclusive but in common with some other messengers essential for physiopathological cell responses. Accordingly, none of these pathways can be too drastically blocked or too much activated without risk. On the other hand, NO is, in many cases, not the exclusive player in neurodegenerative processes but, instead, one of the players of a very complex game. For all the above reasons, a useful future direction could be represented by combined treatments in which NO inhibitors or releasers are mixed with antioxidants and signaling pathway modulators.

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