Redox-modulation of NMDA receptor activity by nitric oxide congeners

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In neurons, nitric oxide (NO·) is produced by neuronal nitric oxide synthase following stimulation of *N*-methyl-D-aspartate (NMDA) receptors and the subsequent influx of Ca^{2+}. NO·, induced in this manner, reportedly plays critical roles in neuronal plasticity, including neurite outgrowth, synaptic transmission, and long-term potentiation (LTP) (1-7). However, excessive activation of NMDA receptors has also been shown to be associated with various neurological disorders, including focal ischemia, epilepsy, trauma, neuropathic pain and chronic neurodegenerative maladies, such as Parkinson's disease, Hungtington's disease and amyotrophic lateral sclerosis (8). The paradox that nitric oxide (NO·) has both neuroprotective and neurodestructive effects may be explained, at least in part, by the finding that NO effects on neurons are dependent on the redox state. This claim may be supported by the recent finding that tissue concentrations of cysteine approach 700 μM in settings of cerebral ischemia (9), levels of thiol that is expected to influence both the redox state of the system and the NO group itself (10).

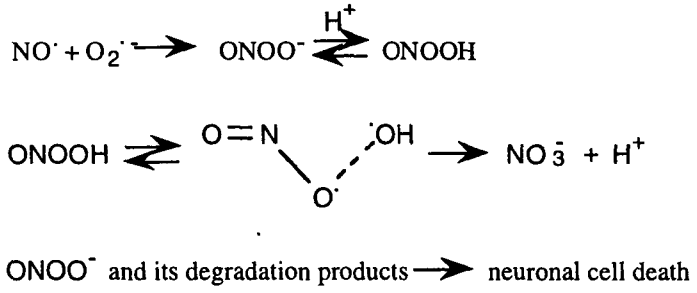
The activity of the NMDA receptor has been shown to be regulated by different allosteric modulators which interact with specific sites (11). These include co-agonist binding sites for glutamate (or NMDA) and glycine, and a redox-modulatory site(s) defined by the existence of critical thiol group(s) (11, 12). Recently, our laboratory and collaborators identified two cysteine residues in the rat NMDAR1 subunit (Cys-744 and Cys-798) mediating redox-modulation of the NMDA receptor-channel complex, although other cysteine residues may also be involved (13). This redox modulatory site(s) controls the frequency of opening of the receptor-coupled ion channel: when oxidized (probably to a disulfide bond), channel activity is downregulated and when chemically reduced, the channel opens more frequently, allowing additional influx of Ca^{2+} following agonist binding.

Excess influx of calcium may cause neuronal cell death (8). One possible contributory mechanism for calcium-mediated neurotoxicity is that an excess rise in neuronal intracellular calcium concentration ($[Ca^{2+}]i$) leads to an increase in constitutive nitric oxide synthase activity, in turn resulting in release of nitric oxide which damages surrounding neurons (14,15). However, several groups have been unable to demonstrate direct toxicity of nitric oxide on

cortical neurons and some have even found that NO donor compounds protect against NMDA receptor-mediated neurotoxicity (16). Similar discordant effects of nitric oxide have been reported in other tissues. To address this paradox, therefore, we studied the NO-related activity by considering its various redox-related states and their distinctive chemistries: NO^+ (nitrosonium cation, with one less electron than NO·), NO· (nitric oxide, with one free electron in its outer orbital and, therefore, a radical species), and NO^- (nitroxyl anion, with one additional electron compared to NO·) (10).

Nitric oxide (NO·)-mediated neurotoxicity

NO· releasing agents, such as *S*-nitrosocysteine (cys-NO; 200 μM) or 3-morpholino-sydnonimine (SIN-1; 1 mM) caused a damage in rat cerebrocortical neurons, which was confirmed by trypan blue exclusion and lactate dehydrogenase (LDH) leakage. The toxicity of cys-NO was inhibited by pre-incubation with superoxide dismutase (SOD) and catalase (CAT), whereas NO· generation was not significanlt changed. This superoxide-dependent toxicity was also confirmed with SIN-1, the N-hydroxy nitrosamine (NONOate), and spermine-NO (17, 18). These events are best rationalized as follows: cys-NO and SIN-1 rapidly liberate nitric oxide (NO·), which in turn reacts with endogenous superoxide ($O_2^{\cdot -}$) to induce neuronal damage through formation of peroxynitrite ($OONO^-$). This pathway is supported by the direct



demonstration of neurotoxic effects of $OONO^-$ on neurons (19). Predictably, SOD/CAT did not prevent the toxicity of $OONO^-$. The neurotoxic effects of peroxynitrite could be due to nitration of tyrosine residues on critical proteins, peroxidation of lipids, and oxidation of sulfhydryls (20, 21). Our previous study showed that $ONOO^-$ decreased NMDA-mediated increases in $[Ca^{2+}]i$, implying no correlation of $ONOO^-$-induced neurotoxicity with change of $[Ca^{2+}]i$ (22). Interestingly, NMDA receptor activation has been reported to lead production of NO· (14) and $O_2^{\cdot -}$(23), and thus presumably would generate $OONO^-$, at least under some conditions. Recent observations that increased nitric oxide synthase (NOS) activity leads to DNA damage and hence stimulation of polyADP-ribose synthase (24), which compromises cell

viability, are consistent with the nitrosative and nitrative actions of $OONO^-$. However, neuronal susceptibility to NO· and/or $O_2^{\cdot-}$ -induced damage could depend on the nature of the neurons and on the environmental conditions. For example, cerebellar granule cells, known to contain high levels of NOS (25, 26), could be more resistant than other neurons to NO-induced cell death (27). We also favor the possibility that lower levels of $OONO^-$ may serve a signalling function.

NO· reacts with thiol groups only at very slow rates under physiological conditions. We also found that NO· itself is not primarily responsible for regulation of NMDA receptor activity, as we found no alteration of NMDA receptor-mediated increase in $[Ca^{2+}]i$ by NO· donor compounds including spermine/NO and diethyamine/NO (monitored by whole-cell recording with a patch electrode or by digital calcium imaging with the dye fura-2) (28). Hence, in our view nitric oxide is not primarily responsible for downregulation of NMDA receptor activity but other redox-related states of the NO group may be involved.

Nitrosonium (NO^+)-mediated neuroprotection

We next examined the effects of several NO^+ equivalents on NMDA receptor-mediated neurotoxicity. Sodium nitroprusside (SNP), which has NO^+ character, significantly protected rat cortical neurons against NMDA-induced neurotoxicity, whereas SNP alone had no significant affect when added to cultures (19). SNP with NO^+ character does not liberate NO· spontaneously in the absence of reductive activation, but will react with thiolate anion (RS^-) on the NMDA redox site(s). Subsequently, a complex shown in figure 1 is formed in which sulfur is bound to nitrogen. This reaction is best rationalized in terms of *S*-nitros(yl)ation.

Downregulation of NMDA receptor activity may occur directly (through formation of the above complex) or by way of subsequent oxidation of the redox site to disulfide (represented by a dashed line in the chemical structure in Fig. 1). Likewise, SNP decreased NMDA receptor activity as demonstrated by whole-cell recording with a patch electrode or by digital calcium imaging with the calcium sensitive dye fura-2 (19).

One-electron transfer processes are required to interconvert redox-related forms of the NO group (10). Thus, to emphasize further that ambient redox conditions will dictate the potential neuroprotective or neurotoxic effects of a given nitroso-compound, we incubated sodium nitroprusside with excess ascorbate or thiol (cysteine or N-acetylcysteine) to show that we could convert nitroprusside to a neurotoxin. These substances act as reducing agents, thereby promoting the evolution of NO· from sodium nitroprusside (i.e., $NO^+ \xrightleftharpoons{e^-}$ NO·), which in turn leads to neurotoxicity by reaction with $O_2^{\cdot-}$ (Fig. 2) (19). As predicted, under our conditions SOD protected neurons against this toxic mechanism by scavenging $O_2^{\cdot-}$ and thus attenuating peroxynitrite formation.

S-nitroso proteins form readily under physiologic conditions and possess biological activities such as endothelial relaxing factor (EDRF)-like effects of vasodilatation and inhibition of platelet aggregation (29). Glutathione is one endogenous thiol that reacts with the NO group to form *S*-nitrosoglutathione, a compound that has been identified in vivo (30); this may help explain the effects of glutathione on the NMDA receptor's redox site (31). Compared to NO·, the relative stability of RS-NO implies a lack of reactivity of the NO group (NO^+) with superoxide (10). Formation of RS-NO at the NMDA receptor's redox modulatory site,

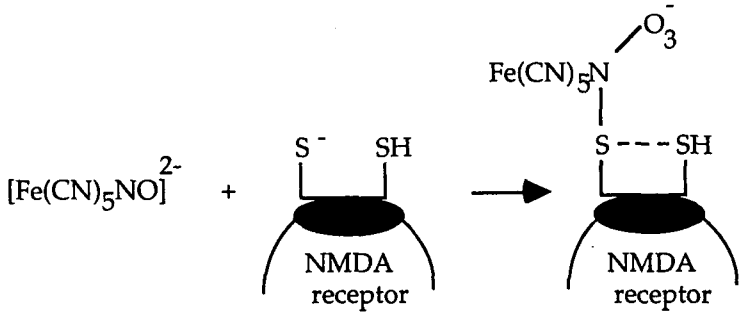


Figure 1. Mechanism of protection from NMDA receptor-mediated neurotoxicity by sodium nitroprusside (SNP). 'Redox site' indicates one or more thiol groups on the NMDA receptor-channel complex comprising a redox modulatory site that can be *S*-nitros(yl)ated, possibly facilitating disulfide bond formation.

therefore, may not only downregulate NMDA-associated ion channel activity and consequently harmful Ca^{2+} influx (16), but may also provide a means to avoid neurotoxic reactions of NO· with superoxide.

Based on the above findings, the ideal NO group donor drug would be one that does not rapidly generate NO·, but rather would react readily with the critical thiol group(s) of the redox modulatory site(s) of the NMDA receptor to inhibit Ca^{2+} influx. We therefore studied nitroglycerin (NTG) as an exemplary compound. Similar to sodium nitroprusside, this drug does not spontaneously liberate nitric oxide to any significant extent, and it is known to react readily with thiol groups forming derivative thionitrites (RS-NO) or thionitrates ($RS-NO_2$) (16, 19).

Using whole-cell recording with patch clamp electrodes and digital calcium imaging with fura-2, we found that nitroglycerin inhibits NMDA-evoked currents and Ca^{2+} influx (16, 19). The effect of nitroglycerin having NO^+ character appears to be mediated by its reactions

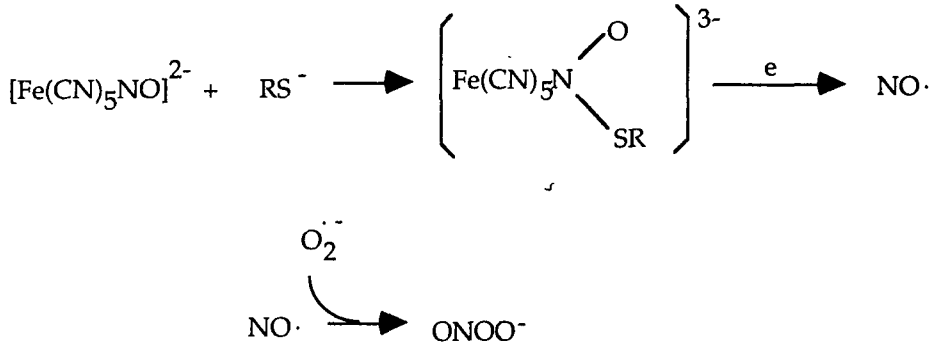


Figure 2. Mechanism of neurotoxicity engendered by sodium nitroprusside (SNP). The NO group of SNP (in the form of NO^+) gains an electron from a donor to liberate nitric oxide (NO·). Nitric oxide then reacts with superoxide anion to generate peroxynitrite. Peroxynitrite or one of its breakdown products is neurotoxic (Lipton et al., 1993).

with thiols of the redox-modulatory sites of NMDA receptors (it was demosntrated by specific alkylation of thiol groups with N-ethylmaleimide (NEM) which completely abrogated the inhibitory effect of nitroglycerin on subsequent NMDA-evoked responses (16).

The finding that nitroglycerin could inhibit NMDA-evoked responses was corroborated by the demonstration that nitroglycerin also significantly ameliorates NMDA-induced neuronal killing in cerebrocortical cultures (16, 19). In addition, preliminary data suggest that high doses of nitroglycerin are neuroprotective in rat models of focal ischemia under conditions of constant systemic blood pressure and cerebral blood flow (32).

Nitroxyl anion (NO^-)-mediated neuroprotection

Recently, we have extended our observations by studying the effects of nitroxyl anion (NO^-), which contains one additional electron compared to NO·. Under physiological conditions, NO^- can be synthesized by the reaction of NO· with Cu(I)-SOD (33). NO^- generated enzymatically in this fashion or by the addition of exogenous NO^- donors was applied to rat cortical neurons in culture while monitoring their NMDA-evoked responses during patch-clamp recording or digital Ca^{2+} imaging with fura-2.

NMDA-evoked increases in $[Ca^{2+}]i$ were inhibited by three distinct NO^- donors. Also, similar concentrations of the NO^- donors consistently ameliorated NMDA receptor-mediated neurotoxicity to a significant degree. These effects of NO^- donors were prevented by oxidation of free thiols by a strong oxidizing reagent 5-5'-dithio(bis-2-nitrobenzoic acid) or alkylation of free thiols by NEM following application of dithiothreitol (DTT). These effects can be explained by the known reaction of NO^- with thiols, which leads to sulfhydryl oxidation to disulfide, through intermediate formation of RSNHOH, in this case at a redox modulatory site of the NMDA receptor (Fig. 3A). The NO^- released from NO^- donors has been known to have energy high enough to react directly with thiols.

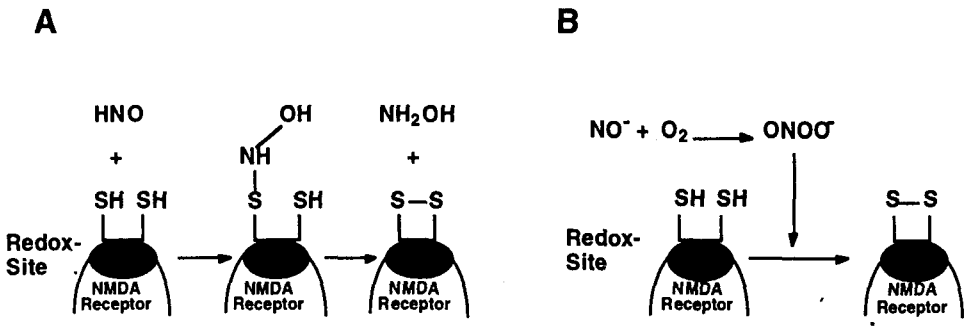


Fig. 3. Mechanism of the action of NO^- on thiol (-SH) groups. A: NO^- in the singlet state. The redox modulatory site of NMDA receptor can be oxidized by NO^-, which may further facilitate disulfide bond formation through intermediate formation of RSNHOH(25, 26). HNO is actually the combined form of NO^- and H^+. B: NO^- in the triplet state. NO^- in this state reacts preferentially with oxygen to generate $ONOO^-$, which oxidizes the redox-modulatory sites of NMDA receptor.

We have further studied if NO^- produced by incubation of NO· donors with Cu(I)-SOD down regualted NMDA-receptor activity. Unlike the NO^- released from NO^- donors, the NO^- produced by Cu(I)-SOD appears to be in the triplet state (a lower energy state of the paired electrons in the outer molecular orbital than the singlet state). This species reacts preferentialy with oxygen (rather than thiol) to generate peroxynitrite, which oxidizes the redox-modulatory sites of NMDA receptor and decreases the activity of the receptor (Fig. 3B).

Taken together, the actions of the NO group are determined by its redox state. Nitric oxide appears to be associated, at least in part, with neuronal injury caused by excessive activation of the NMDA receptor. In this story peroxynitrite forms from the reaction of NO· with superoxide anion ($O_2^{\cdot -}$) and results in severe neuronal cell damage. Pharmacologically,

thus, NMDA receptor-mediated neurotoxicity may be prevented by drugs containing the NO group in alternative redox states. The mechanism for neuroprotective effects appears to involve *S*-nitrosylation or oxidation of critical thiols to disulfide bonds in the NMDA receptor's redox modulatory site(s), which downregulates channel activity. NO· itself reacts only very slowly with thiol (-SH) groups (34-36), consistent with the lack of effect of NO· on NMDA receptor-mediated responses under physiological conditions. It is inferred that the alternative redox-activated states, NO^+ and NO^-, are likely to be the species directly reactive with thiol groups. Under certain conditions, neuroprotection may be achieved by limiting peroxynitrite formation from NO·, for example, with nitric oxide synthase inhibitors and superoxide scavengers, or by fostering NO group transfer (of NO^+ or NO^-) to the critical thiol groups of the NMDA receptor's redox modulatory site(s). It is becoming increasingly evident that in addition to NMDA receptors, biological activities of many other proteins containing critical cysteine residues can be regulated by *S*-nitrosylation and oxidation, in a sense similar to the type of control exerted by phosphorylation of critical tyrosine or serine groups. This chemical reaction may be a novel and ubiquitous pathway for the molecular control of protein function by potential reactive sulfhydryl centers.

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