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Commentary

Nitric oxide: Foe or friend to the injured brain?

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Over the past 6 years or so, the reactive gas nitric oxide (NO) has become established as a diffusible messenger mediating cell–cell interactions throughout the body, including immune cell-mediated cytotoxicity, inhibition of platelet aggregation, smooth muscle relaxation, and neuronal signaling (1). Appreciation that NO serves as a neuronal messenger was triggered by the discoveries that cultured cerebellar neurons release an NO-like muscle relaxing factor, and that brain neurons contain a constitutive form of the enzyme responsible for synthesizing NO from arginine, nitric oxide synthase (cNOS) (2, 3). Neuron-derived NO regulates smooth muscle tone and the behavior of other central neurons as an "aberrant transmitter" (3), differing from more conventional neurotransmitters in being able to diffuse freely from the point of synthesis to intracellular target sites in neighboring cells and being independent of vesicular release, membrane receptors, or lipid cell boundaries. While the normal neuronal actions of NO have not been fully delineated, NO has been implicated specifically in some forms of synaptic plasticity, including both long-term potentiation and long-term depression (4, 5).

A key role for NO has also been postulated in the pathogenesis of brain damage following acute insults, such as hypoxia–ischemia (6). This hypothesis represents a consequential new twist on an older premise that the cytotoxic overstimulation of neuronal glutamate receptors, especially of the *N*-methyl-D-aspartate (NMDA) type, contributes to hypoxic–ischemic neuronal loss (7, 8). Such NMDA receptor-mediated "excitotoxicity" depends on extracellular Ca^{2+} and is likely initiated by excess Ca^{2+} influx through the NMDA receptor-gated channel (9). The linkage of neuronal NMDA receptor stimulation to activation of neuronal cNOS in an extracellular Ca^{2+}- and calmodulin-dependent fashion (10, 11) led Dawson *et al.* (12) to propose that NO formation by neuronal cNOS mediates NMDA receptor-mediated neurotoxicity.

This idea received strong support from their experiments showing that NMDA receptor-mediated neuronal death in cortical cell cultures could be blocked by four different maneuvers: (*i*) pharmacological inhibition of NOS; (*ii*) addition of hemoglobin, which complexes with free NO; (*iii*) deletion of the NO precursor, L-arginine, from the bathing medium; and (*iv*) prior selective destruction of the small neuronal subpopulation containing high concentrations of cNOS, previously referred to as "NADPH-diaphorase"-containing cells. The idea was reinforced by the finding that the glutamate receptor-mediated neurotoxicity of the human immunodeficiency virus type 1 coat protein gp120 (13) could also be blocked by inhibition of NO formation (14). Most recently, the same laboratory has reported that calcineurin inhibition by the immunosuppressant FK506 results in increased phorbol ester-induced cNOS phosphorylation, reduced cNOS catalytic activity, and reduced neuronal vulnerability to NMDA-induced neurotoxicity (15).

This latest observation may have broad implications for understanding the normal regulation of cNOS activity. As predicted by its sequence, neuronal cNOS can be phosphorylated by protein kinases, specifically protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase, and cAMP-dependent protein kinase, resulting in an inhibition of catalytic activity (3). Dawson *et al.* (15) proposed that NMDA receptor-mediated Ca^{2+} influx may activate calcineurin to dephosphorylate cNOS, acting synergistically with Ca^{2+}/calmodulin to enhance cNOS activation. They cited unpublished data indicating that cGMP-dependent protein kinase can also phosphorylate cNOS. Although guanylyl cyclase has a macroscopically different distribution in brain than cNOS, perhaps enough guanylyl cyclase is present in cNOS-containing neurons to provide a negative feedback loop: NO formation inducing local cGMP formation, cNOS phosphorylation, and resultant cNOS inhibition.

Additional work will be needed to establish that calcineurin-induced cNOS dephosphorylation regulates neuronal cNOS activity *in vivo*. The study of Dawson *et al.* (15) was carried out in 293 human kidney cells overexpressing cloned neuronal cNOS; extension to primary cultured neurons or brain slices and eventually to the intact brain will be desirable. Furthermore, the authors caution that FK506 has known actions other than calcineurin inhibition and increases the phosphorylation of proteins other than NOS.

If calcineurin-induced cNOS dephosphorylation does indeed enhance brain neuronal cNOS activity *in vivo*, calcineurin inhibition might be an attractive therapeutic goal in the setting of focal brain ischemia (e.g., stroke) and other forms of brain injury involving NMDA receptor-mediated excitotoxicity. As Dawson *et al.* point out, both FK506 and another immunosuppressant, cyclosporin A, are already used in humans to suppress organ rejection after transplant surgery. One study has raised the possibility that FK506 may reduce global ischemic brain injury in liver transplant patients (16), although the small size of this study and the paucity of other evidence supporting a role of NMDA receptor-mediated neurotoxicity in animal models of global ischemia (17) are caveats. A second therapeutic strategy might be to enhance cNOS phosphorylation by one or another kinase, although some indirect data has implicated kinases in the pathogenesis of ischemic brain injury. It may turn out that manipulating the cNOS phosphorylation state cannot be accomplished selectively enough to form a practical therapeutic strategy. Even so, the protective effect of FK506 *in vitro* provides additional rationale for examining other means of attenuating neuronal cNOS activity as a treatment for focal brain ischemia. Encouraging results have been reported with competitive NOS inhibitors in some animal models of stroke (18–20).

However, as highlighted by the study of Wink *et al.* (21) in the present issue, not all available data support a role of neuronal NO formation in pathological neuronal death. Wink *et al.* describe the use of spontaneous NO-releasing compounds, NONOates, to elucidate a cytoprotective effect of NO against cell death induced by exposure to hydrogen peroxide and superoxide in transformed fibroblast cell cultures or primary cultures of mesencephalic neurons.

These are exciting, provocative observations; follow-up experiments will be needed to determine their generality. Studies will be needed to compare results

obtained with NONOates with results obtained with the NO-generating compounds used in other studies, such as *S*-nitrosocysteine, 3-morpholinosydnonimine (SIN-1), and *S*-nitroso-*N*-acetylpenicillamine (SNAP). Reliance on generator compounds is necessitated by the instability of NO itself, but the possibility that the generator compound directly influences resultant effects must be considered. For example, interpretation of data gathered by using the NO generator nitroprusside is confounded by the recent finding that attenuation of the NMDA receptor-mediated Ca^{2+} influx by the ferrocyanide portion of the molecule can be independent of NO formation (22).

Critical involvement of NO formation in NMDA receptor-mediated neurotoxicity or hypoxic–ischemic neuronal death is also challenged directly by several negative studies that have failed to find that NOS inhibitors reduce NMDA-induced neuronal death (23–26) or focal ischemic brain injury *in vivo* (27). Further, the notion that NO is the toxic effector of NMDA receptor-mediated toxicity does not fit in any simple fashion with the observation that the small cortical neuronal subpopulation containing high concentrations of cNOS (the neurons staining for NADPH-diaphorase) are themselves selectively resistant to NMDA-induced death (9).

Can these disparate results be reconciled? There is every reason to think so, given the multiplex degrees of freedom inherent in the unfolding details of the brain NO system. Data provided by Snyder and colleagues (3, 6) provide compelling evidence that the neuronal NO formation triggered by brief, intense NMDA receptor stimulation has the potential to be a critical mediator of subsequent excitotoxic death. On the other hand, the above negative studies suggest that NO formation is not always required for a phenotypically similar, rapidly triggered NMDA receptor-mediated death. Currently undefined differences in experimental conditions may lower the toxic potential of NO formation enough that other injury mechanisms predominate. Cell death is a nonspecific endpoint, common to all injury processes. If several injury cascades occur simultaneously, only blockade of the most powerful cascade—the cascade most capable of producing lethal injury after a given insult—will lead to an increase in cell survival.

The precise mechanisms by which NO overformation can kill neurons remain to be clarified but likely overlap with the mechanisms by which macrophage-derived NO kills bacteria or neoplastic cells. NO complexes with iron–sulfur groups within key enzymes necessary for DNA replication and mitochondrial energy production (1). In addition, NO reacts with superoxide to form peroxynitrite, a potent inducer of lipid peroxidation and other free radical-mediated degenerative reactions (28, 29) (Fig. 1). This latter mechanism is consistent with the observation that rapidly triggered, NMDA receptor-mediated excitotoxicity on cultured cortical neurons can be partially attenuated by 21-aminosteroid radical scavenger compounds (31). However, NO formation is not the only pathway connecting cellular Ca^{2+} overload with free radical production. Another pathway, for example, is Ca^{2+} activation of phospholipase A_2, liberating arachidonic acid and leading to enhanced production of free radicals by lipoxygenase and cyclooxygenase pathways. Inhibition of lipoxygenase pathways with nordihydroguaiaretic acid or baicalein reduces slowly-triggered excitotoxic death in cortical cell cultures (ref. 32; also unpublished results).

Three main factors might attenuate the prominence of an NO-mediated neurotoxicity in a given system.

(i) Reduced NO generation (or increased NO degradation). The simplest reason why NO-mediated injury might make a relatively minor contribution to NMDA-induced neurotoxicity in a given system would be a relatively low level of NMDA receptor-mediated NO release, for example because of a paucity of neu-

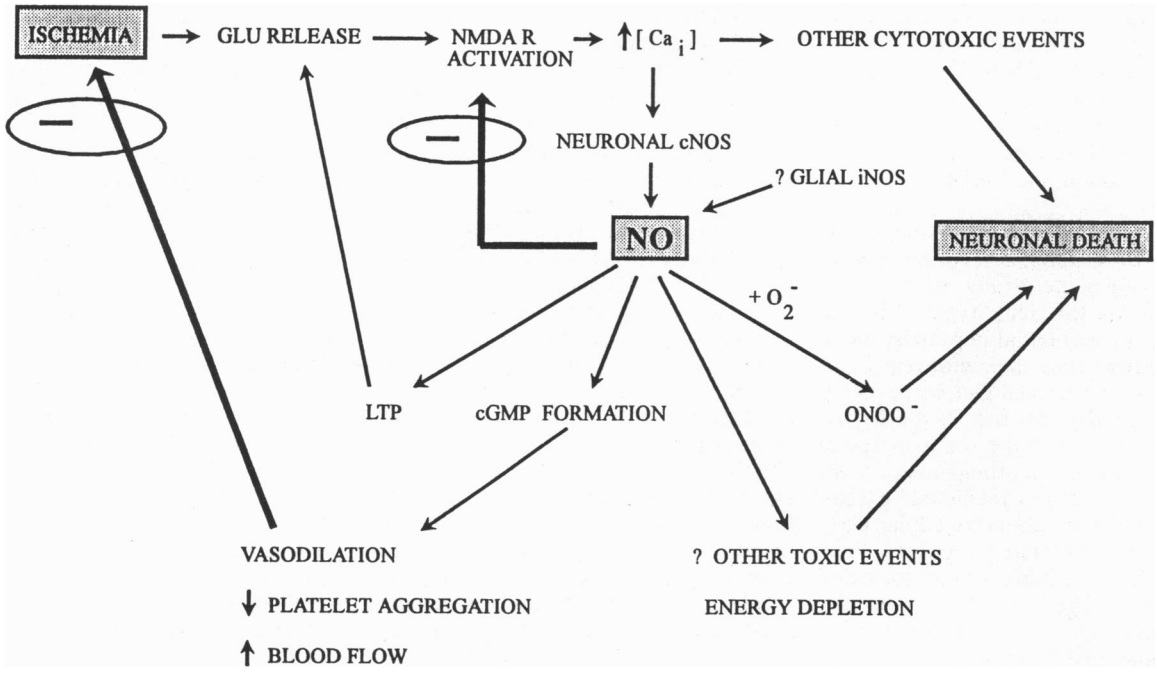

FIG. 1. NO: foe or friend of the ischemic brain? Speculative diagram summarizing some possible relationships between brain ischemia, NMDA receptor overactivation, NO formation, and cell injury. NO is postulated to be cytotoxic primarily through combination with superoxide to form the destructive free radical, peroxynitrite. The alternative possibility that NO itself induces injury—for example, by impairing mitochondrial energy production—is also indicated. (Note that these two possibilities have opposite predictions for the effect of superoxide removal on injury—e.g., by superoxide dismutase. Superoxide removal, which reduces NO-induced cytotoxicity (28, 29), reduces peroxynitrite formation but prolongs the half-life of NO itself.) Two cytoprotective "brakes" on the injury cascade are shown: (*i*) NO-mediated NMDA receptor down-modulation and (*ii*) NO-mediated vasodilation and reduced platelet aggregation, leading to increased blood flow. The diagram additionally incorporates a recent speculation that NO produced by induction of astrocytic iNOS (perhaps by cytokines released after injury) may be a key contributor to NMDA receptor-mediated neurotoxicity (30).

rons containing high concentrations of cNOS. An interesting corollary might then be the possibility that stimulation of other, nonneuronal sources of NO might bring out an NO-mediated component of NMDA receptor-mediated excitotoxicity.

We recently have found that exposure to γ interferon combined with either interleukin 1β or lipopolysaccharide can induce NOS expression in cultured cortical astrocytes [presumably the inducible form (33), or iNOS, as this expression is blocked by cycloheximide] (30). Prior treatment of cortical cell cultures (containing both neurons and glia) with these cytokines potentiates NMDA receptor-mediated neurotoxicity, and this potentiation is blocked by N^G-nitro-L-arginine—in other words, causes our system to behave in a fashion similar to that described by Dawson *et al.*, in terms of NO and excitotoxicity. The cultures used by Dawson *et al.* (12) were treated after 4 days *in vitro* to inhibit cell division, making it unlikely that astrocyte iNOS plays a large role in their system. Nonetheless, our observations support a speculation that cytokine stimulation of astrocytes, leading to enhanced NO production by astrocytic iNOS, may contribute to an NO-mediated component of neuronal damage in certain disease states (Fig. 1).

(ii) Reduced susceptibility to NO cytotoxicity. The vulnerability of a given cell to NO-mediated cytotoxicity might be expected to depend on numerous internal characteristics, including dependence on iron–sulfur-containing enzymes, total energy stores, ability to withstand altered ion homeostasis, free radical defenses, and availability of reactive oxygen species. It is possible that the transformed fibroblast cells or mesencephalic neurons studied by Wink *et al.* (21) are intrinsically less vulnerable to damage by peroxynitrite than other central neurons. For example, mesencephalic neurons may need special defenses to handle the free radicals generated by dopamine metabolism.

(iii) Counterbalancing cytoprotective effects of NO. A third basis for reconciling disparate observations regarding the neuroprotective efficacy of NOS inhibition is provided by the idea discussed above that NO formation may have cytoprotective effects that counterbalance its cytotoxic effects (21). Of note, recent evidence indicates that NO can downmodulate the NMDA receptor, although whether this effect is mediated by oxidation of the NMDA receptor redox site (34) or by other actions (35, 36) is unsettled.

Besides exerting direct cytoprotective effects on ischemic brain parenchyma, NO may also influence injury *in vivo* by dilating arterioles and inhibiting platelet aggregation, thus improving tissue perfusion and attenuating the ischemic insult (Fig. 1).

In summary, a plethora of variables and the inherent complexity of NO biology hinder present efforts to define the effects of NO upon the injured brain. Recent studies have given us important new information and direction but in doing so have intensified the fundamental issue of whether NO production is helpful or deleterious in the setting of cerebral ischemia. Do we want to augment or attenuate brain NO production in patients suffering a stroke? The answer to this question may prove to be as multifaceted as NO itself.

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