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Commentary

Potent neuroprotectants linked to bifunctional inhibition

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Neuronal death after ischemic or traumatic injury is mediated, in large part, by excitotoxicity. Originally, it was thought that cell damage by ischemia/reperfusion and other forms of neuronal insults was caused by calcium-mediated activation of phospholipases and proteases, leading to release of free fatty acids and their metabolites, the concomitant generation of reactive oxygen species, and the degradation of cytoskeletal proteins. In an effort to limit the formation of reactive oxygen species in human disease and provide functional sparing of brain tissue, pharmaceutical research in the past focused on the development of free-radical scavengers (1). Unfortunately, many of these approaches were met with disappointment. From recent studies, it is now clear that an important coupling exists between glutamate release, calcium influx, and enhanced production of reactive oxygen species, such as superoxide anion, hydrogen peroxide, hydroxyl radical, and nitric oxide (NO). Of these reactive oxygen species, recent efforts have focused on the reaction product of NO and superoxide anion, peroxynitrite ($ONOO^-$), an oxidant with potentially devastating cellular effects. Drug development has concentrated primarily on strategies to limit $ONOO^-$ formation by developing NO synthase (NOS) inhibitors (2) or superoxide dismutase mimetics (3). Discoveries in this area leading to potentially therapeutically useful agents have also been a disappointment. Chabrier *et al*. (4) report a bifunctional agent, BN 80933, which exerts the dual effect of inhibition of NO formation and scavenging of reactive oxygen species. BN 80933 provides significant neuroprotection even when administered after the neurologic insult.

NO is an important biologic messenger molecule that mediates physiologic activities as diverse as regulation of vascular tone and blood pressure, control of platelet activation, regulation of neurotransmitter release, and mediation of the cytotoxic actions of activated macrophages, as well as acting as the nitrergic transmitter of the peripheral nervous system, to name a few (5). NOS exists in three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (6). Because of its ability to modulate neurotransmitter release and reuptake, mitochondrial respiration, DNA synthesis, and energy metabolism, it is not surprising that excessive NO generation is neurotoxic. Under conditions in which NO is produced for sustained periods, such as after iNOS induction in the central nervous system, dysregulation of normal physiologic activities by NO likely contributes to neuronal dysfunction and subsequent neuronal death. However, acute toxicity, such as that observed after stroke or trauma, seems to require production of superoxide anion concomitantly with NO. In the presence of superoxide anion, NO becomes a potent neurotoxin because of the formation of the potent oxidant $ONOO^-$. It is most likely that $ONOO^-$ mediates the toxic activities of excessive NO production (7). $ONOO^-$ is a lipid-permeable molecule with a wider range of chemical targets than NO. It can oxidize proteins, lipids, RNA, and DNA. Neurotoxicity elicited by $ONOO^-$ formation may have several components. Peroxynitrite inhibits the function of manganese superoxide dismutase (8), as well as the mitochondrial respiratory chain (9), which could lead to increased superoxide anion formation and increased $ONOO^-$ formation. Peroxynitrite efficiently modifies and breaks DNA strands and inhibits DNA ligase, which potentiates DNA damage (10). DNA strand breaks initiate DNA repair mechanisms, including activation of poly (ADP ribose) polymerase (PARP). In particular, PARP activation can result in a rapid drop in energy stores that likely contributes to neuronal cell death (11). This pathway is thought to play a particularly important role in ischemia/reperfusion injury (12, 13). Thus, strategies aimed at reducing NO, superoxide anion, and $ONOO^-$ formation might be particularly beneficial. Other potentially important mediators of neuronal cell death include activation of caspase-dependent apoptotic pathways (14, 15).

Depending on its source, NO may be toxic or protective to the brain under ischemic conditions (16, 17). Clarification of the action of NO in focal ischemia was ascertained through pharmacological and genetic approaches. nNOS-derived NO plays a prominent role in the early phases of neuronal injury through *N*-methyl-D-aspartate glutamate-receptor-mediated calcium-dependent activation of nNOS (16). NO generated by iNOS plays a role in the late phase of neuronal injury, because it is induced after stroke and exacerbates excitotoxic injury (17). Because NO generated from eNOS is crucial in maintaining cerebral blood flow, eNOS-derived NO is neuroprotective; inhibiting eNOS can have drastic deleterious effects on the nervous system (18). To limit neurotoxicity, selective inhibition of either or both nNOS and iNOS is warranted (17, 19). However, finding agents that selectively inhibit only these two isoforms *in vivo* has proven difficult.

Free-radical scavengers, from vitamin E to lazaroids, have been studied in both *in vitro* and *in vivo* models of excitotoxicity, ischemia, and traumatic brain injury (20–22), but they have not yielded very significant protection. For free-radical scavengers to be effective therapies, they must readily reach the target site of radical generation, exceed the concentration of *in situ*-generated free radicals, and have a very fast rate constant for reaction with radicals (23). Most free-radical scavengers fall short of these requirements.

The disappointing performance of NOS inhibitors or free-radical scavengers may be caused, in part, by the fact that monotherapy leaves the other radical species free to act alone and elicit toxicity. Consistent with this concept, inhibition or deletion of NOS coupled with free-radical scavenging results in a synergistic sparing of neurons. For instance, primary neuronal cultures generated from nNOS null mice are protected further from *N*-methyl-D-aspartate neurotoxicity by application of superoxide dismutase (24). Rats treated with combined subthreshold concentration of the NOS inhibitor, N^G-nitro-L-arginine, and the antioxidant/superoxide scavenger, di-tert-butyl-hydroxybenzoic acid, had a greater reduction in infarct volume after middle cerebral artery occlusion (25) than those treated with either agent alone. These studies suggest that NO and free radicals are involved and interact in

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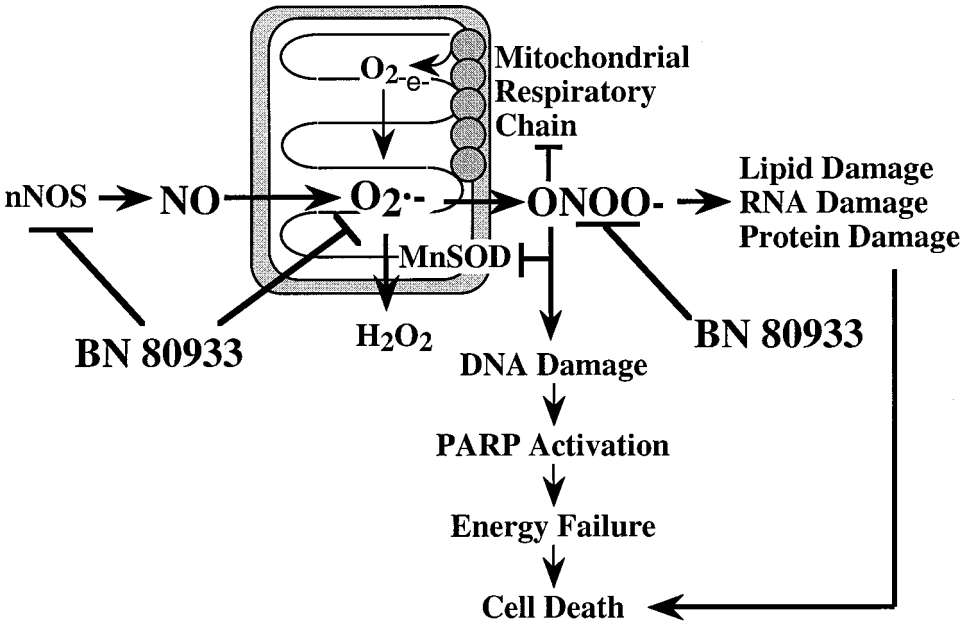

FIG. 1. Pathways of neuroprotection by the bifunctional inhibitor BN 80933. MnSOD, manganese superoxide dismutase.

synergy in ischemia/reperfusion injury. Chabrier *et al*. (4) take these observations further with the development and description of a pharmacologic agent that effectively inhibits NOS and scavenges free radicals (Fig. 1).

Trolox- or vitamin E-related compounds have been modified in the past to increase their solubility and bioavailability with subsequent increased efficacy (26, 27), and attempts have been made to develop relatively selective nNOS and iNOS inhibitors. The work by Chabrier *et al*. (4) takes a clever approach. Instead of attempting to make a slightly better antioxidant or NOS inhibitor, they coupled an antioxidant (a Trolox derivative) via a piperidine linker to an NOS inhibitor (thiophene amidine). This chemistry resulted in a molecule, BN 80933, with dual function. BN 80933 not only selectively inhibits nNOS, but it also effectively scavenges reactive oxygen species. An added benefit may be its increased bioavailability and subcellular targeting. This linkage created an extremely effective therapeutic agent with a broad therapeutic index in multiple models of neuronal injury. *In vivo*, this agent not only does not affect eNOS or vascular tone, which makes it a significantly improved nNOS inhibitor, but it also seems to scavenge reactive oxygen species advantageously. Most important is that the protection conferred by this agent is sustained; thus, it can be given 4–8 h after injury and still provide significant neuroprotection. This approach, which one could envisage being employed with alternative targets, is particularly innovative. For instance, the coupling of PARP inhibitors with caspase inhibitors might create a particularly attractive neuroprotectant; such an agent would be expected to inhibit both necrotic and apoptotic cell-death pathways. The addition of a linker yields a chemical composition that is readily patentable. Thus, one can take two separate known compounds and create a third proprietary agent. This strategy could be exploited to the advantage of pharmaceutical companies and most importantly to the benefit of patients.

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