Another Facet of Nitric Oxide: Reduction of Toxic Zinc Influx Through Voltage-Gated Channels


Summary. The neurotoxicity of zinc, released from nerve terminals during global ischemia, may contribute to the delayed death of certain selectively vulnerable neuronal populations. A likely first event in zinc-induced neuronal death appears to be its permeation across the plasma membrane, largely through voltage- and agonist-gated calcium channels. Considering the possibility that cellular Zn$^{2+}$ overload might be lethal for reasons similar to cellular calcium overload, we tested the hypothesis that Zn$^{2+}$ neurotoxicity might be mediated by activation of neuronal nitric oxide synthase (NOS), an event implicated in the pathogenesis of excitotoxic neuronal death. However, physiologically relevant concentrations of zinc (30–100 nM) had no effect on NOS activity, while 100–300 μM Zn$^{2+}$ actually inhibited NOS activity in solution. The addition of extracellular Zn$^{2+}$ did not affect NOS activity in cultured murine neocortical neurons, assessed by measuring cyclic guanosine 5’-monophosphate (cGMP) levels, and the concurrent addition of NOS inhibitors did not alter Zn$^{2+}$-induced neuronal death (cultures were exposed to 300–500 μM Zn$^{2+}$ for 5 min under depolarizing conditions; neuronal degeneration was assessed 24 h later). Rather, addition of the nitric oxide (NO) precursor, l-arginine, or the diazeniumdiolate (NONOate) NO donors (DEA/NO) or 1-propanamine,3-(2-hydroxy-2-nitroso-1-propylhydrazino) NONOate (PAPA/NO) markedly reduced Zn$^{2+}$-induced neuronal death and produced a dose-dependent block of high K$^+$-stimulated cellular $^{45}$Ca$^{2+}$ uptake. The oxidizing agents thimerosal and 2,2’-dithiodipyridine (DTDP) also reduced K$^+$-stimulated cellular $^{45}$Ca$^{2+}$ uptake, while alkylation of thiols by pretreatment with N-ethylmaleimide (NEM) blocked the reduction of $^{45}$Ca$^{2+}$ uptake by NO donors. These results suggest that Zn$^{2+}$-induced neuronal death is not mediated by the activation of NOS; rather, any available NO may attenuate Zn$^{2+}$ neurotoxicity, in part through a down-modulation of Zn$^{2+}$ entry through voltage-gated Ca$^{2+}$ channels.

Introduction

Zn$^{2+}$ is present at high concentrations in the central nervous system and is localized in synaptic boutons, usually of glutamatergic neurons [3, 8, 10]. It is released with nerve cell activity; extracellular concentrations may reach the 100-μM range in regions such as the mossy fiber terminals in the CA3 region of the hippocampus [5, 7, 16, 17]. The basis of Zn$^{2+}$ neurotoxicity has not been established, but likely involves Zn$^{2+}$ entry into neurons through both agonist and voltage-gated Ca$^{2+}$ channels. Several types of evidence suggest that voltage-gated Ca$^{2+}$ channels are the major route by which excessive Zn$^{2+}$ can enter neurons under depolarizing conditions. Depolariza-
tion enhances accumulation of $^{65}\text{Zn}^{2+}$ in cultured neurons (H.S. Ying et al., unpublished results), as well as $[\text{Zn}^{2+}]$, and neuronal vulnerability to $\text{Zn}^{2+}$-induced death [18]. $\text{Zn}^{2+}$ entry and $\text{Zn}^{2+}$-induced neuronal death can be attenuated by the application of voltage-gated $\text{Ca}^{2+}$-channel antagonists, particularly the L type (H.S. Ying et al., unpublished results) [4, 11, 18].

Because $\text{Zn}^{2+}$ probably enters neurons through many of the same routes as $\text{Ca}^{2+}$, it is possible that the neuronal death induced by cellular $\text{Zn}^{2+}$ overload might result from some of the same mechanisms responsible for $\text{Ca}^{2+}$-overload-induced neuronal death, such as occurs following glutamate-receptor over-stimulation. A prominent mechanism in the latter category is the over-activation of nitric oxide synthase (NOS) leading to the production of toxic levels of nitric oxide (NO). While extremely high (100 $\mu$M) concentrations of $\text{Zn}^{2+}$ have been reported to inhibit brain-tissue NOS in crude enzyme preparations [1, 15], activation of NOS by the 1–100 nM $\text{Zn}^{2+}$ concentrations that may be relevant to physiological or even pathophysiological conditions [11] can be considered.

We exposed primary cultures of murine neocortical neurons to $\text{Zn}^{2+}$ under depolarizing conditions to test the hypothesis that $\text{Zn}^{2+}$ neurotoxicity, like excitotoxic neuronal death, is substantially mediated by the activation of neuronal NOS (nNOS).

**Materials and Methods**

**Cell Culture and Exposure to Drugs**

Mixed neocortical cultures were prepared from mouse cortices as previously described [9]. Cultures were maintained in a 37 °C, humidified incubator in a 5% CO$_2$ atmosphere. All experiments were performed after 14 days in vitro.

Mixed cultures of neocortical neurons and glia were exposed to $\text{Zn}^{2+}$ (300–500 $\mu$M ZnCl$_2$) for 6 min in room air in the presence of 45 mM KCl. The exposure was performed in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered control salt solution (HCSS: 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl$_2$, 1.8 mM CaCl$_2$, 10 mM NaOH, 10 $\mu$M glycine, 20 mM HEPES, 5.5 mM glucose, pH 7.4). HCSS with increased potassium was made by substituting KCl for NaCl. Since exposure to high potassium could be expected to induce secondary glutamate release, we included the competitive N-methyl-D-aspartate (NMDA) antagonist D-4-(3-phosphopropyl)piperazine-2-carboxylic acid (D-CPP) (100 $\mu$M) in the exposure medium to block NMDA receptor-mediated toxicity. The NO donors diethylamine NONOate (DEA/NO) or 1-propanamine, 3-(2-hydroxy-2-nitroso-1-propylhydrazino) NONOate (PAPA/NO) were made up at 100–300 mM in 10 mM NaOH, stored at 4°C and used the same day. The stock solution was diluted into neutral pH buffer immediately before use. Cell death was estimated 20–24 h after exposure to drugs by phase-contrast microscopy and quantified by measuring lactate dehydrogenase (LDH) present in the bathing medium [13]. To determine the percentage of neurons dying, LDH efflux from treatment conditions was divided by LDH efflux from sister cultures treated for 24 h with 300 $\mu$M NMDA, a treatment that kills virtually all the neurons.