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Release of intracellular Zn^{2+} in cultured neurons after brief exposure to low concentrations of exogenous nitric oxide

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Abstract Several studies have shown intracellular Zn^{2+} release and concomitant cell death after prolonged exposure to exogenous NO. In the present study, we investigated whether cortical neurons briefly exposured to exogenous NO would demonstrate similar levels of intracellular Zn^{2+} release and subsequent cell death. Cortical neurons were loaded with the Zn^{2+} selective fluorophore FluoZin-3 and treated with various concentrations of the NO generator, spermine NONOate. Fluorescence microscopy was used to detect and quantify intracellular Zn^{2+} levels. Concomitant EDTA perfusion was used to eliminate potential effects of extracellular Zn^{2+}. Neurons were perfused with the heavy metal chelator TPEN to selectively eliminate Zn^{2+} induced fluorescence changes. A significant increase of intracellular fluorescence was detected during a 5 min perfusion with spermine NONOate. The increase in intracellular Zn^{2+} release appeared to peak at 1 μM spermine NONOate (123.8 ± 28.5%, increase above control $n = 20$, $P < 0.001$). Further increases in spermine NONOate levels as high as 1 mM failed to further increase detectable intracellular Zn^{2+} levels. The NO scavenger hemoglobin blocked the effects of spermine NONOate and the inactive analog of the spermine NONOate, spermine, was without effect. No evidence of cell death induced by any of the brief treatments with exogenous NO was observed; only prolonged incubation with much larger amounts of exogenous NO resulted in significant cell death. These data suggest that in vivo release of NO may cause elevations of intracellular Zn^{2+} in cortical neurons. The possibility that release of intracellular Zn^{2+} in response to NO could play a role in intracellular signaling is discussed.

Keywords Intracellular Zn^{2+} · FluoZin-3 · Primary culture · Metallothionein

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Introduction

Zn^{2+} is an essential micronutrient and is required for the normal development and function of the nervous system (Takeda 2000). There is increasing evidence for a direct signaling function for Zn^{2+} at different levels of signal transduction through regulatory mechanisms that are still largely unknown (Beyersmann and Haase 2001; Maret 2001). For example, incubation of PC12 rat pheochromocytoma cells with Zn^{2+} led to an increase of the cellular cGMP concentration

(Watjen et al. 2001). Several laboratories have investigated the activation of mitogenic signaling pathways by Zn^{2+} (Zago et al. 2005; Kohda et al. 2006; Uzzo et al. 2006). Since most of the experimental evidence comes from in vitro experiments, it is not yet generally accepted that Zn^{2+} is indeed a signaling molecule in vivo. In particular, the mechanism by which intracellular Zn^{2+} activates various signaling pathways is still unknown.

The interacting pools of intracellular Zn^{2+} that presumably exist in neurons include a free cytosolic pool, Zn^{2+} associated with cytosolic metalloprotein buffers and Zn^{2+} sequestered in intracellular organelles (Colvin et al. 2003). Using high affinity Zn^{2+} selective fluorophores, the free cytosolic pool is estimated to be at most 1 nM (Krezel and Maret 2006). The organelle pool includes Zn^{2+} sequestered in synaptic vesicles, mitochondria and the Golgi (Cole et al. 1999; Eide 2006; Sensi et al. 2003; Varea et al. 2001). Though different intracellular Zn^{2+} pools are recognized, how these pools interact with each out is still unclear, especially in neurons.

Small changes in intracellular free Zn^{2+} are likely buffered by metallothioneins (MTs) (Krezel and Maret 2006). Metallothioneins are ubiquitous, sulphur-rich, low-molecular-weight proteins with a high affinity for essential trace metals, such as Zn^{2+}. Three of four known isoforms are identified in the nervous system (Aschner et al. 1997). The expression of MT-I and MT-II are mainly localized in glial cells (Aschner et al. 1997); on the other hand, MT-III is mostly present in neurons (Masters et al. 1994). In response to oxidized glutathione (GSSG), Zn^{2+} can be released from MTs (Maret and Vallee 1998). MTs seem to act as a cytosolic Zn^{2+} buffer but the physiological conditions of Zn^{2+} release are mostly unknown. Experimental evidence has shown that long duration exposure to millimolar levels of exogenous NO results in increased levels of intracellular free Zn^{2+} (Berendji et al. 1997). Research has suggested that NO induces Zn^{2+} release from MT by S-nitrosylation and disulfide formation both in vitro and in vivo (Kroncke et al. 1994; Zhang et al. 2004).

Nitric oxide (NO) is a transient molecule that contributes to a wide variety of physiological and pathological processes. NO activates guanylyl cyclase to generate cGMP, an important second messenger molecule (Ignarro 1999). Inducible nitric oxide synthase (iNOS) becomes active in the presence of cytokines or bacterial LPS and elevates NO production in several human diseases including ischemic events (Kroncke et al. 1998) and can produce micromolar concentrations of NO for prolonged periods of time (Laurent et al. 1996). The lowest concentration of NO that has been detected in neurons by microelectrodes is approximately 20–40 nM (Dobrucki et al. 2000). While under pathological conditions such as ischemic neuronal injury, the concentration of NO in cells can become 2–4 μM (Dobrucki et al. 2000).

Research efforts have focused on understanding the relationship between increased NO levels and Zn^{2+} release from MT. When cultured neurons were treated with high concentrations of NO, peroxynitrite is produced, which leads to the release of Zn^{2+} from MT. The increase of free Zn^{2+} can result in more peroxynitrite production (Bossy-Wetzel et al. 2001). The accumulated peroxynitrite and ROS can oxidize and activate the phosphorylation of p38 MAPK to trigger downstream signaling pathways. All these events lead to the activation of caspase to cause cell death (Bossy-Wetzel et al. 2001). Another pathway that can be induced by increased Zn^{2+} is the 12-lipoxygenase (12-LOX) pathway. The activation of 12-LOX can mediate arachidonic acid (AA) metabolism to increase ROS production (Zhang et al. 2004). In these experiments, high concentrations of NO were used to cause Zn^{2+} release. Under physiological conditions, endogenous NO is much lower, thus whether Zn^{2+} release occurs under physiological conditions has not been demonstrated.

As previous experiments have used long term exposures to NO to increase intracellular Zn^{2+}, little is known about what will happen to intracellular free Zn^{2+} levels after brief physiological exposure to NO. Therefore, we studied short term, low concentration exposures to extracellular NO, that were not neurotoxic, to determine if the release of intracellular Zn^{2+} was still a feature of such experimental manipulations. We demonstrated that cultured neurons after short term

exposure to exogenous NO showed a concentration-dependent increase in intracellular free Zn^{2+} levels. We found no evidence of cell death induced by any of the brief treatments with exogenous NO. This suggests that the released Zn^{2+} may have important physiological functions, such as a signaling molecule to trigger or modulate downstream pathways.

Materials and methods

Materials

Spermine NONOate, hemoglobin and spermine were obtained from Calbiochem. FluoZin-3 and Pluronic acid F-127 were obtained from Molecular Probes. MTT was obtained from Sigma. All other chemicals used were of the highest purity available.

Preparation of primary cortical neuron

Primary culture of embryonic (E 17-18) Sprague-Dawley rat cortical neurons was performed as described previously (Colvin 2002). The appropriate concentration of cells was plated on the center of a rectanglar coverslip (22 × 40 mm, Warner Instruments, No.1 Cover Glass) surrounded by vacuum grease (Dow Corning). The coverslip was coated with polyethylenimine (50% solution, Sigma) diluted 1:1,000 in borate buffer and allowed to attach to the surface surrounded by the grease dam at 37°C, 5% CO_2 in supplemented Minimal Essential Medium (MEM) solution {MEM solution (Gibco BRL) supplemented with 10 mM sodium bicarbonate, 2 mM L-glutamine, 1 mM pyruvate, 20 mM KCl and 10% (v/v) heat inactivated fetal bovine serum}. After 24 h, the coverslip was switched to Neurobasal supplemented with 0.5 mM L-glutamate and 2% B-27 and maintained in the same condition.

Fluorescence microscopy

The cells were incubated for 30 min at 37°C with 10 μM FluoZin-3 (Molecular Probes) with Pluronic acid F-127 (Molecular Probes) in 1:1 ratio in Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 10 mM Glucose; pH 7.4). Post incubation, neurons were washed once in Locke's buffer, then the coverslip was mounted in a sealed perfusion chamber (Warner Instruments, Model RC-30 HV). Neurons were examined with a 60X oil objective (Plan Apo/1.40 oil DIC, Nikon) using an inverted epifluorescence microscope (Nikon, Diaphot 300) and FITC filter (Chroma 41001). Fluorescent images were captured with a CCD camera (Spot, RT ES, model 9.1 Monochrome w/ IR-6). Neutral density filters were applied to decrease photobleaching. For each coverslip, three different regions were observed. After perfusion with each treatment, the same three regions were observed in sequence and the fluorescence images captured. The control was obtained by perfusing the cells with Locke's Buffer containing 100 μM EDTA for the entire length of the experiments. The perfusion with EDTA resulted in a decrease in fluorescent intensity, this most likely due to Zn^{2+} efflux from the neurons and photobleaching. After 5 and 10 min perfusion with 100 μM EDTA alone, the fluorescence intensity decreased to 90.69 ± 0.50% (mean ± SEM) and 84.05 ± 0.56% respectively compared to the beginning fluorescence level. For this reason, data obtained from co-perfusion with EDTA were corrected by the formula: $F_{corrected} = F_{observed}/Ratio_{EDTA}$ ($Ratio_{EDTA}$ was the ratio of fluorescent intensity changes before and after the EDTA perfusion. The values were 0.91 and 0.84 for 5 min and 10 min EDTA perfusions, respectively), to exclude the interference of EDTA. The rate of perfusion for all treatments was about 1 ml/min.

Fluorescent images were captured and saved as 16 bit TIFF uncompressed files with no post processing. Using MetaMorph (Version 4.6) software, the cell body of each neuron was outlined (region of interest) and the average pixel intensity was obtained. The background average pixel intensity of the same size on the same image was also measured. The background average pixel intensity was subtracted from the cellular average pixel intensity.

To compare fluorescent changes across different coverslips, fluorescence intensity was normalized by transforming the data to F/F_0. F_0 was the

average of the background corrected pixel intensity of all the cells after perfusion with Locke's buffer in the three different areas observed on each coverslip. F was the background corrected cellular pixel intensity of each cell at various times after different experimental treatments. Also, to compare the effect of different concentrations of exogenous NO, we calculated a normalized ΔF_{max}. To determine the maximum response to each concentration of NO, the highest increase of the three measurements during perfusion with spermine NONOate was selected (ΔF_{max}). The average of ΔF_{max} was converted into percentage increase, and then was plotted.

MTT (methyl-thiazolyl-diphenyl-tetrazolium) assay

High-density cortical neuronal cultures grown in 24-well plates were used for this experiment. During the experiment, they were treated with various concentrations of spermine NONOate or $ZnCl_2$ in Locke's buffer (pH 7.4) for the same time period as cells on coverslips exposed to the perfusion protocol. After the treatments, Neurobasal medium was added to replace the solutions. 24 h later, the medium was removed and 500 µl of MTT (Sigma) solution (1:10 diluted in Locke's buffer from 5 mg/ml stock solution) was added and incubated for 1 h at 37°C. Then the solution was removed and 1 ml DMSO was added and incubated for 5 min at 37°C. The whole medium was transferred to a cuvette and read in the spectrophotometer (Cary 50 Probe, Varian) at 550 nm and 650 nm wavelength.

Statistical analysis

All data plots and appropriate statistical analyses were generated using GraphPad Prism 4.0 (Graph Pad Software). The data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA and post hoc multiple comparison test. The statistical significance of differences was set as $P < 0.05$.

Results

Visualization of intracellular Zn^{2+} changes in primary cortical neuronal cells with FluoZin-3 AM

Intracellular Zn^{2+} levels were visualized by the high affinity Zn^{2+} indicator FluoZin-3 AM. 25 µM pyrithione, a Zn^{2+} ionophore, and 100 µM Zn^{2+} was used as a positive control to load cells with large amounts of Zn^{2+}. Images were captured before and after Zn^{2+} and pyrithione perfusion followed by perfusion with 100 µM tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN). As expected, the observed intracellular fluorescence intensity increased rapidly and significantly after perfusion with Zn^{2+} and pyrithione. The intracellular fluorescence levels obtained in Fig. 1B would presumably reflect fluorophore saturation. Fluorescence decreased to a level below that of the resting FluoZin-3 fluorescence (Fig. 1A) after perfusion with 100 µM TPEN for 5 min (Fig. 1C). TPEN is a cell-permeable metal chelator, which should reduce free intracellular Zn^{2+} to very low levels below the detection limit of FluoZin-3.

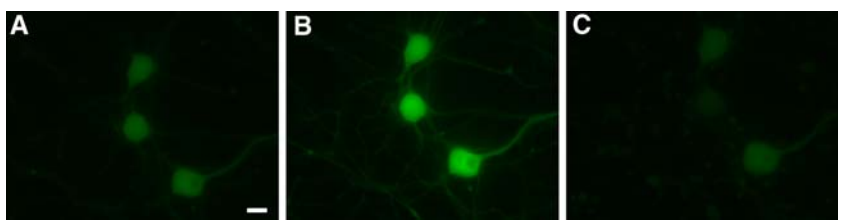


Fig. 1 Effects of perfusion with Locke's Buffer, 100 µM Zn^{2+} and 25 µM Pyrithione, and 100 µM TPEN on FluoZin-3 fluorescence intensity: Coverslips containing cortical neurons were incubated with 10 µM FluoZin-3 and 0.01% pluronic acid (1:1 mixture) for 30 min and mounted in a perfusion chamber. (**A**) 5 min after perfusion with Locke's buffer. (**B**) 5 min after perfusion with Zn^{2+} and Pyrithione. (**C**) 5 min after perfusion with TPEN. Legend bar = 10 µm

Presumably then, the fluorescence levels obtained after TPEN perfusion reflect cellular autofluorescence. The decrease in fluorescence observed after TPEN addition confirmed that the increase in fluorescence caused by Zn^{2+} and pyrithione perfusion was most likely due to increased cellular uptake of Zn^{2+}. The cellular fluorescence showed a uniform distribution in all neurons. There was no evidence of compartmentalization of the fluorophore in subcellular organelles.

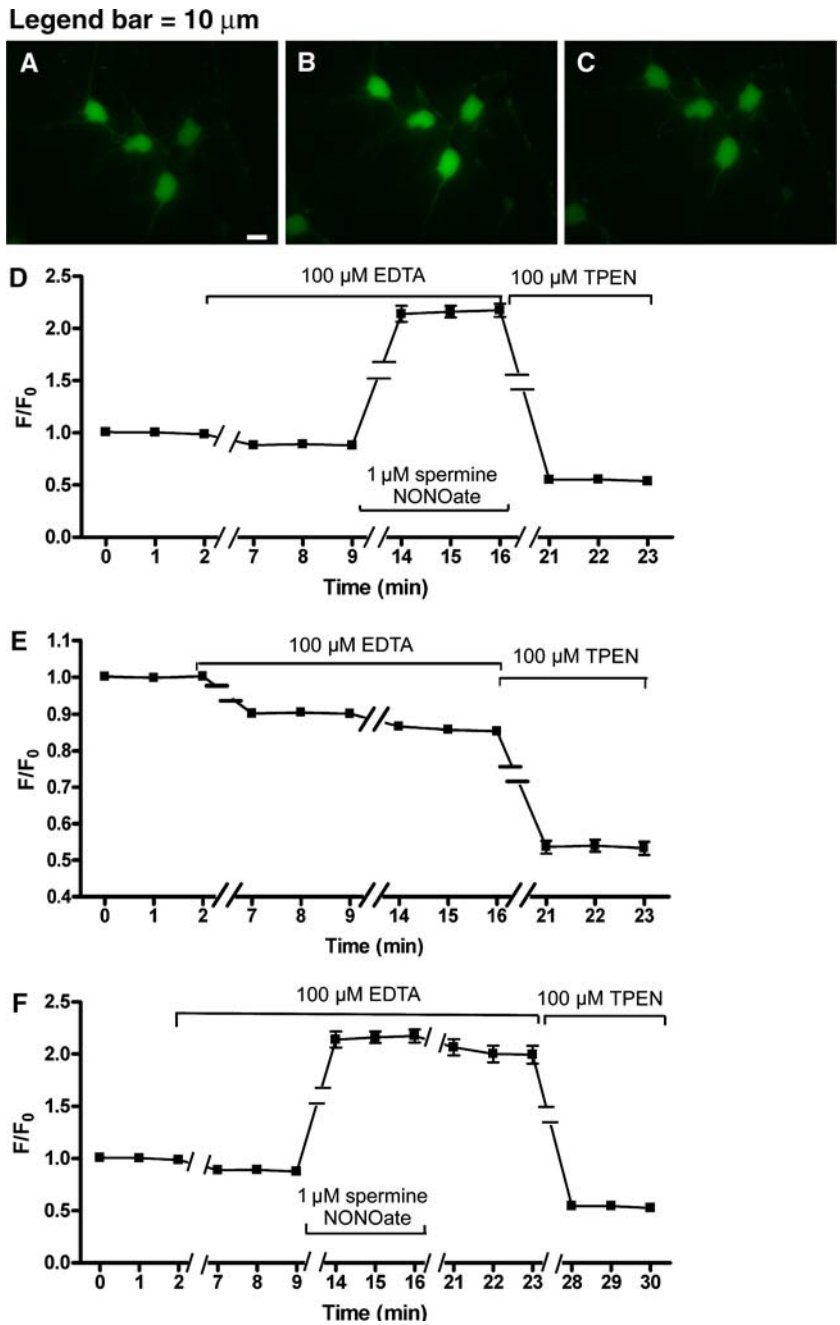
Addition of extracellular spermine NONOate causes release of intracellular Zn^{2+}

To investigate the effects of extracellular NO on intracellular free Zn^{2+} levels, the NO generator spermine NONOate, which has a 1:2 stoichiometry for NO production and a $t_{½}$ of 39 min at 37°C, was used. There is as much as 1 μM contaminating Zn^{2+} present in the Locke's buffer (determined by ICP-MS, data not shown). This extracellular Zn^{2+} could enter the neurons to cause an intracellular fluorescence increase. To avoid this, 100 μM EDTA was included in the perfusion solutions to eliminate any possible Zn^{2+} influx, without altering extracellular Ca^{2+} or Mg^{2+} concentrations. Fig. 2A–C show representative images, while Fig 2D–F show average fluorescence changes observed. The cellular fluorescence was increased during perfusion with 1 μM spermine NONOate (Fig. 2B). After perfusion with 100 μM TPEN for 5 min (Fig. 2C), the cellular fluorescence decreased. Fig. 2D illustrates the effect of perfusion with 100 μM EDTA; followed by 1 μM spermine NONOate and finally 100 μM TPEN, averaged over 20 neurons. The effect of spermine NONOate was significant ($P < 0.001$) when compared to cells prior to spermine NONOate perfusion. Since these effects were obtained in the presence of extracellular 100 μM EDTA (Zn^{2+} influx blocked), the observed increase in intracellular Zn^{2+} must represent the release of Zn^{2+} from intracellular stores previously inaccessible to FluoZin-3. Figure 2E shows that the perfusion of 100 μM EDTA alone did not cause an increase of fluorescence. Thus, perfusion with 100 μM TPEN and the subsequent rapid drop in fluorescence indicated that the increased fluorescence signal was due to changes in intracellular free Zn^{2+}. To demonstrate that the reversed fluorescence is caused only by TPEN, after perfusion with spermine NONOate, EDTA alone was perfused, but no dramatic decrease of fluorescence was observed. Thus, after washout of spermine NONOate, a persistent increase in intracellular free Zn^{2+} was observed. Fluorescence intensity decreased only when TPEN was perfused subsequently (Fig. 2F).

Next, the inactive analog of spermine NONOate (spermine) was tested. The result showed that the cellular fluorescence intensity did not increase after perfusion with 1 μM spermine and 100 μM EDTA (Fig. 3A). The fluorescence intensity decreased because of Zn^{2+} efflux and/or photobleaching. This result confirmed that the observed fluorescence increases were likely caused by NO released from spermine NONOate. Was the NO released from spermine NONOate responsible for the observed increase in cellular fluorescence? To test this, hemoglobin, (an NO scavenger that can react with NO at nearly diffusion-limited rates to produce nitrate and iron-nitrosylhemoglobin), was used (Herold et al. 2001). The results showed that the fluorescence intensity did not increase after perfusion with 10 μM hemoglobin and 1 μM spermine NONOate (Fig. 3B). Again, the fluorescence intensity decreased because 100 μM EDTA was present in the perfusion solution. These data confirmed that the fluorescence change observed was caused by NO produced by spermine NONOate only.

Dose response analysis of extracellular spermine NONOate addition

To determine if the effect of spermine NONOate was dose dependent, the neurons were treated with various concentrations of spermine NONOate. The treated neurons showed a concentration-dependent increase in fluorescence (Fig. 4A). The fluorescent change observed with 0.5 μM spermine NONOate was significant compared to before the perfusion with spermine NONOate (35.35 ± 9.61% mean ± SEM, $P < 0.001$, $n = 18$). The increase in fluorescence intensity peaked at 1 μM spermine NONOate (123.8 ± 28.47%, $P < 0.001$, $n = 20$), and decreased as the spermine NONOate concentration was further elevated.



2 μM spermine NONOate caused an increase of 108.6 ± 28.45% ($P < 0.001$, $n = 18$) and 5 μM increased the fluorescence by only 67.17 ± 12.27% ($P < 0.001$, $n = 20$). Neurons were perfused with Zn^{2+} and pyrithione after the perfusion with spermine NONOate. The results show that although 1 μM spermine NONOate produced a maximal fluorescence response, relative to addition of exogenous NO generator, the fluorophore was not saturated, as evidenced by the increase in fluorescence observed when Zn^{2+} and pyrithione were subsequently added (Fig. 4B). This signal was completely reversed by perfusing with 100 μM TPEN. Higher concentrations of

◀**Fig. 2** Effect of brief perfusion with 1 μM spermine NONOate: Coverslips containing cortical neurons were prepared as in Fig. 1. (**A**) 5 min after perfusion with 100 μM EDTA. (**B**) 5 min after perfusion with 1 μM spermine NONOate. (**C**) 5 min after perfusion with 100 μM TPEN. Images were quantified and normalized to F/F_0 (see methods). (**D**) The fluorescent changes after perfusion with 1 μM spermine NONOate. After 5 min perfusion with Locke's buffer, neurons were perfused with 100 μM EDTA. Then, neurons were perfused for 5 min with 1 μM spermine NONOate. Finally, the neurons were perfused with 100 μM TPEN for 5 min. (**E**) The fluorescent changes after perfusion with 100 μM EDTA. After 5 min perfusion with Locke's buffer, neurons were perfused with 100 μM EDTA. Then, neurons were perfused for another 5 min with 100 μM EDTA. Finally, the neurons were perfused with 100 μM TPEN for 5 min. (**F**) The washout experiment. After 5 min perfusion with Locke's buffer, neurons were perfused with 100 μM EDTA. Then, neurons were perfused for 5 min with 1 μM spermine NONOate. Neurons were perfused with 100 μM EDTA for another 5 min. Finally, the neurons were perfused with 100 μM TPEN for 5 min. Each point represents the mean±SEM ($n = 20$ neurons) from three different coverslips

spermine NONOate (1 mM) were tested, but saturation of fluorophore was not observed since the addition of Zn^{2+} and pyrithione produced further fluorescence increase (Fig. 4C).

Brief treatment with extracellular spermine NONOate did not cause cell death

To determine whether the treatments used for the experiments described were neurotoxic, a cell death assay with MTT was performed. The high-density cortical neuronal cultures were incubated under various conditions and times followed by 100 μM TPEN for 5 min, then the medium was changed back to Neurobasal medium and the neurons were incubated overnight at 37°C. The next day the MTT assay was performed to quantify viable cells. The result showed that neurons did not demonstrate significant cell death after brief exposure to either 1 μM or 1 mM spermine NONOate compared to the same period treated with Locke's buffer (101.75 ± 3.91%, $n = 3$; 91.84 ± 0.96%, $n = 3$, respectively). Since the treatment with Locke's buffer for 3 h did cause a significant decrease in cell viability (78.04 ± 2.64%, $P < 0.01$, $n = 3$), longer time exposures to spermine NONOate were compared to the same period treated with Locke's buffer. No significant decrease in cell viability occurred after treatment with 1 μM spermine NONOate (95.99 ± 2.76%, $n = 3$) for 3 h, but 1 mM spermine NONOate for 3 h did show a significant decrease in cell viability (58.85 ± 5.34%, $n = 3$, $P < 0.001$). Incubation in 1 mM Zn^{2+} overnight showed nearly complete death of all neurons compared to neurons incubated in Neurobasal medium (5.23 ± 0.95%, $n = 6$, $P < 0.001$).

Discussion

In this study, evidence is provided that exposure of cultured neurons to a broad range of concentrations of exogenous NO resulted in a rapid and persistent increase in intracellular free Zn^{2+}. Previous studies (Horn et al. 2002) have shown that treatment of cultured neurons with 4–10 μM NO produced an increase in intracellular Ca^{2+} levels as detected by Fura-2 fluorescence. Since it is now well established that several ''classical'' Ca^{2+} indicators are sensitive to both Ca^{2+} and Zn^{2+}, is it possible that the fluorescence changes observed in the present study are the result of increased intracellular Ca^{2+} and not Zn^{2+}? This seems highly unlikely since the fluorophore used in the present study is quite Zn^{2+} selective and is insensitive to millimolar levels of Ca^{2+}. In addition, all observed increases in FluoZin-3 were reversible with 100 μM TPEN, which shows a high degree of selectivity for metals (including Zn^{2+}) over Ca^{2+}. Thus, we conclude that the fluorescence changes observed in the present study were due to only changes in intracellular free Zn^{2+}. Similar results (i.e., an increase in intracellular Zn^{2+}) have been observed after the administration of NO generators to the dorsal hippocampus of intact rats (Cuajungco and Lees 1998) and these increases were prevented by preadministration of TPEN. It is interesting to note that the Fura-2 fluorescence changes observed by Horn and colleagues could be due to changes in either Ca^{2+}, Zn^{2+} or both ions. A more careful study using various flurophores and specific metal chelators will be needed to resolve this issue.

The source of released Zn^{2+} in the present study is clearly intracellular, since incubation with

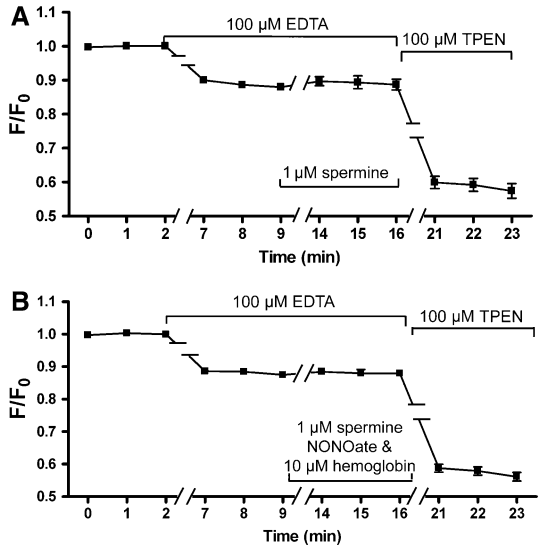


Fig. 3 Effect of brief perfusion with spermine and NO scavenger. Coverslips were prepared as in Fig 1. (**A**) After 5 min perfusion with Locke's buffer, neurons were perfused with 100 μM EDTA. Then, neurons were perfused for 5 min with 1 μM spermine. Finally, the neurons were perfused with 100 μM TPEN for 5 min. (**B**) After 5 min perfusion with Locke's buffer, neurons were perfused for 5 min with 100 μM EDTA. Then, neurons were perfused for 5 min with 10 μM hemoglobin and 1 μM spermine NONOate. Finally, the neurons were perfused with 100 μM TPEN for 5 min. Each point represents the mean ± SEM (n = 20 neurons) from three different coverslips.

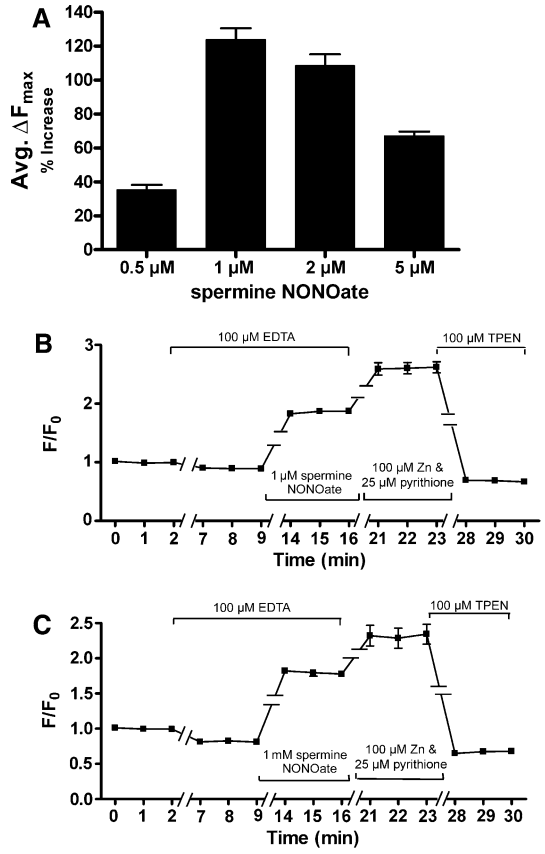


Fig. 4 (**A**) Dose dependent effect of spermine NONOate. Each bar represents the average maximal response after perfusion with 0.5, 1, 2 and 5 μM spermine NONOate. (**B**) Neurons were perfused with 100 μM EDTA for 5 min. Next, neurons were perfused with 1 μM spermine NONOate for 5 min. Then, neurons were perfused with 100 μM Zn^{2+} and 25 μM pythione. Finally, neurons were perfused with 100 μM TPEN. Each point represented the mean ± SEM (n = 18 neruons) from three different coverslips. (**C**) Neurons were perfused with 100 μM EDTA for 5 min. Next, neurons were perfused with 1 mM spermine NONOate for 5 min. Then, neurons were perfused with 100 μM Zn^{2+} and 25 μM pythione. Finally, neurons were perfused with 100 μM TPEN. Each point represented the mean ± SEM (n = 20 neruons) from three different coverslips

100 μM extracellular EDTA had no effect on the observed increase in fluorescence (see Fig. 2). However, it was observed that the maximal response of FluoZin-3 to exogenous NO did not represent saturation of the fluorophore, since addition of pyrithione/Zn^{2+} could further increase the observed fluorescent signal. Thus, the rise in intracellular Zn^{2+} had characteristics of a ceiling phenomenon. What could limit the rise in intracellular Zn^{2+}? One possibility would be compartmentalization of FluoZin-3 as has been reported (Devinney et al. 2005), however, no evidence of compartmentalization was observed in our experiments (see Figs. 1 and 2). Another possibility is that the intracellular levels of NO are limited in some way. Several studies now suggest that NO exerts much, if not all of its actions, in the form of S-nitrosothiols especially S-nitrosoglutathiones (GSNOs). It has been shown that GSNO can react with MT in a transnitrosation reaction causing the release of Zn^{2+} (Chen et al. 2002). Recently it has been shown that most cells (including neurons) contain GSNO reductase activity (Jensen et al. 1998; Shah et al. 2007), one of the more interesting being protein disulfide isomerase (PDI) activity (Sliskovic et al. 2005). Thus, neurons are likely to self-limit the levels of intracellular GSNO produced by exogenous NO administration, producing an apparent ''ceiling''

effect. The observed decline in intracellular Zn^{2+} release at 2 and 5 μM spermine NONOate (see Fig. 4) could then be the result of increasing GSNO reductase activity. Finally, it is possible that available Zn^{2+} for release is limited and the ''ceiling'' effect might represent depletion of intracellular pools. It is not known for certain which MT isoforms are predominately expressed in cultured neurons (Aschner et al. 1997), thus it is possible that small pools of MT-III (which is particularly sensitive to transnitrosation (Chen et al. 2002) might exist in cultured neurons and be depleted of Zn^{2+} under the conditions produced in these experiments (See Fig. 4). It was observed that 1 mM NO generator caused increases in intracellular Zn^{2+}, similar to that observed by exposure to 1 μM NO generator (see Fig. 4). It is quite possible that whatever factors were causing a decline in the Zn^{2+} response when NO was raised from 1 to 5 μM would be overwhelmed by additional actions of NO. It should be noted however that exposure to 1 mM NO still did not produce saturation of FluoZin-3.

Previous studies have investigated the toxic effects of NO on cultured cells and neurons (Zhang et al. 2004; Bossy-Wetzel et al. 2001). The present study shows that brief exposures (5 min) to even 1 mM NO generator were not toxic. This observation is in agreement with previous studies that showed that NO toxicity could be at least partially prevented by early chelation of Zn^{2+} but not after a certain amount of time had elapsed (Zhang et al. 2004). The present study showed that 1 mM NO generator was toxic when exposed to cultured neurons for 3 h, but had no effect when the exposure was only 5 min. During long term exposure to NO, it as been suggested that peroxynitrite is formed (Bossy-Wetzel et al. 2001) and increased Zn^{2+} acts to activate 12-LOX and p38 MAPK (Zhang et al. 2004; Bossy-Wetzel et al. 2001) activities leading to apoptotic cell death. It is clearly evident from the present study that after short term exposure (5 min) to physiological concentration (1 μM) of NO, (with concomitant increase in intracellular Zn^{2+}) these cell death pathways are not activated. Thus, this raises the interesting possibility that increases in intracellular free Zn^{2+} caused by NO, might have a signaling function in neurons. However, it is important to note that for a signaling function to exist, a highly effective mechanism to lower intracellular free Zn^{2+} must exist also. Such mechanisms have yet to be characterized in neurons.

There are many published papers which have characterized the actions of Zn^{2+} on various signal transduction pathways. For example, the supplement of physiological levels of Zn^{2+} induced phosphorylation of MAPK to regulate AP-1 and NF-kappa activation in PC-3 human prostate cancer cells (Uzzo et al. 2006). In another study, 100 μM Zn^{2+} initiates the phosphorylation of p70 S6 kinase and glycogen synthase kinase 3beta in SH-SY5Y neuronblastoma cells (An et al. 2005). Zn^{2+} was used to induce modifications in Na^+/H^+ exchange and pyruvate kinase activity through protein kinase C in isolated mantle/gonad cells of Mystilus galloprovincialis (Kaloyianni et al. 2005). On the other hand, previous studies showed that Zn^{2+} suppressed IL-6 synthesis by inhibiting phospholipase C and phospholipase D (Hatakeyama et al. 2002).

In summary, evidence was provided that briefly exposing cultured neurons to physiological concentrations of exogenous NO resulted in persistent increases in intracellular free Zn^{2+} levels. In light of the observation that no significant neuronal death accompanied the increase in intracellular free Zn^{2+}, it seems likely that such increases could play a signaling role in neurons. The present study provides new evidence of crosstalk between NO and intracellular Zn^{2+}, but more research needs to be done to discover exact role that NO induced increases in intracellular free Zn^{2+} might play in neuronal functions.

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