Differential mechanisms of nitric oxide- and peroxynitrite-induced cell death

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ABBREVIATIONS: DETA/NO, diethylenetriamine NONOate, (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)-amino]-diazen-1-ium-1,2-diolate; Sper/NO, spermine NONOate, (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)-butyl]-amino]diazen-1-ium-1,2-diolate; SIN-1, 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; DMEM-F12, 1:1 Dulbecco’s modification of Eagle medium:Ham’s F-12 nutrient mixture; cyclic GMP, cGMP, cyclic guanosine 3,5-monophosphate; IBMX, 3-isobutyl-1-methylxanthine; SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; zVADfmk, carbobenzoxy-valyl-alanyl-aspartyl(β-methyl ester)-fluoromethylketone; IGF-1, insulin-like growth factor-1; LY294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PI3K, phosphatidylinositol 3-kinase; APAF-1, apoptosis-inducing factor-1.
Abstract

Nitric oxide (NO) contributes to cellular degeneration in various disorders, particularly in the nervous system. NO targets cell proteins such as soluble guanylyl cyclase (sGC), but its detrimental effects are generally attributed to its reaction product with superoxide, peroxynitrite. To understand the mechanisms of NO-induced cell stress, we studied the effects of NO donors, diethylenetriamine and spermine NONOate, and the peroxynitrite donor, SIN-1, in SH-SY5Y and NG108-15 neuroblastoma cells. All three compounds induced a dose- and time-dependent decrease in viable cells, which was not blocked by the sGC inhibitor ODQ. The two NONOates were ~15-fold more potent in SH-SY5Y than in NG108-15 cells, whereas the EC$_{50}$ of SIN-1 in SH-SY5Y and NG108-15 cells were in the same order. This led us to conclude that the mechanisms of NO and peroxynitrite did not converge. This was supported by our other findings. NONOates induced DNA fragmentation and an increase in cellular caspase-3 activity that preceded the gradual decline in cell viability. In contrast, SIN-1 induced a transient decline in ATP levels and delayed loss of cell viability with no significant increase in caspase-3 activity or DNA laddering. Moreover, post-treatment with insulin(-like growth factor-1) inhibited caspase-3 activation and loss of cell viability in NONOate- but not in SIN-1-exposed cells. These findings suggest that NO is a potent toxin independent of peroxynitrite formation.
Nitric oxide (NO) has emerged as an important signaling molecule and neurotransmitter. The highest level of NO formation is in the brain, where NO can be generated in vascular, neuronal and glial cells by the calcium-activated endothelial and neuronal nitric oxide synthase (NOS) isoforms or the calcium-independent, inducible NOS. The formation of NO is also a major factor contributing to the loss of neurons in ischemic stroke, demyelinating diseases and other neurodegenerative disorders (Heales et al., 1999). The best characterized NO target is soluble guanylyl cyclase, but NO can react with many other proteins containing iron-sulfur clusters or thiols, forming S-nitrosylated products (Stamler et al., 2001). As well, NO reacts with catecholamines to form 6-nitroderivatives (d'Ischia and Costantini, 1995).

NO itself is a free radical, but in combination with superoxide it forms the very reactive peroxynitrite anion (ONOO\(^-\)). Many of the detrimental effects of NO have been attributed to ONOO\(^-\), because this molecule is able to nitrate and oxidize protein residues as well as catecholamines, DNA and lipids, thereby affecting cellular homeostasis (Torreilles et al., 1999). An increase in tissue 3-nitrotyrosine content has been considered evidence for ONOO\(^-\)-induced changes. Increased levels of protein nitration have been observed in cerebral infarct zones and Lewy bodies as well as in lesions in multiple sclerosis, Alzheimer’s, Huntington’s and ALS (Samdani et al., 1997; Torreilles et al., 1999). In addition, animal models of neurodegeneration have provided \textit{in vivo} evidence for a role of NO synthases and protein nitration (Itzhak et al., 2000; Wu et al., 2002). However, there is some doubt about ONOO\(^-\) being the causal agent of tyrosine nitration (Pfeiffer et al., 2000; Espey et al., 2002). Also, additional harmful factors besides NO were present in the above models. Therefore, we asked the question whether NO itself can be detrimental in absence of ONOO\(^-\) formation.

The recent emergence of compounds that release NO in a controlled manner, in particular the NONOate class, is a great advancement to the study of NO and ONOO\(^-\) effects (Keefer et al., 1996; Feelisch, 1998). Previously, studies were conducted using nitroprusside or
S-nitrosothiols, such as S-nitroso-N-acetyl-DL-penicillamine and S-nitrosogluthathione. The release of NO from the latter type is determined by cellular enzyme activity and redox status (Feelisch, 1998; Haddad et al., 1994), both of which vary depending on cell type. Furthermore, S-nitrosothiols are known to modify proteins by transnitrosation in absence of any NO release (Feelisch, 1998). Nitroprusside produces not only NO but also cyanide, giving it additional toxicity (Feelisch, 1998). In addition, NO effects have often been derived from the outcome of NOS activation and/or inhibition, even though multiple other mechanisms were activated at the same time (Itzhak et al., 2000; Wu et al., 2002).

In the present study, we aimed to distinguish pure NO and ONOO\(^{-}\) effects in order to understand the involvement of ONOO\(^{-}\) formation in NO-induced neuronal damage. Therefore, we compared the effects of three NO donors: DETA/NO and Sper/NO of the NONOate class, and SIN-1. Although SIN-1 is referred to as an “NO donor”, it generates NO and superoxide in equimolar amounts, thereby producing ONOO\(^{-}\) (Feelisch, 1998; Ischiropoulos et al., 1995). Sper/NO and DETA/NO both release NO according to first order kinetics, with half-lives of 39 min and 20 h, respectively (Keefer et al., 1996). The effects of these NO donors were assessed in two neuroblastoma cell lines, human dopaminergic SH-SY5Y and mouse neuroblastoma x rat glioma NG108-15 cells, maintained in absence of growth and differentiation factors. We hypothesized that, if NO-induced cell death involved ONOO\(^{-}\) formation, the effects of DETA/NO and Sper/NO would be similar to those of SIN-1 in any cell type. To the contrary, our results provide evidence that NO is a potent toxin independent of ONOO\(^{-}\) formation.
MATERIALS AND METHODS

Chemicals. DETA/NO, Sper/NO, SIN-1 and ODQ were obtained from Cayman Chem. Co. (Ann Arbor, MI), α-methyl-p-tyrosine and SB203580 from Fisher Scientific (Pittsburgh, PA), 3-aminobenzamide from Calbiochem (San Diego, CA), zVADfmk from Promega Corp. (Madison, WI), recombinant human IGF-1 from R&D Systems (Minneapolis, MN), insulin (from bovine pancreas, ~27 U/mg) and wortmannin from Sigma (St. Louis, MO) and LY294002 from Promega Corp. (Madison, WI) or Biomol (Plymouth Meeting, PA). Unless specified otherwise, all other chemicals were from Fisher or Sigma.

Cell culture. Cell lines, including human dopaminergic neuroblastoma, SH-SY5Y (ATCC #CRL-2266), mouse neuroblastoma x rat glioma, NG108-15 (ATCC #HB-12317), and rat adrenal pheochromocytoma, PC12 (ATCC #CRL-1721), were grown in adherent culture on Cell* tissue culture plastics (Sarstedt, Newton, NC). Cells were maintained in DMEM-F12 (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 1% penicillin-streptomycin-amphotericin B (GIBCO BRL, Gaithersburg, MD) at 37°C, 5% CO₂ and >95% humidity. For experimental purposes, cells were seeded in multiwell plates at 8 x10⁴ cells/cm² and grown for 18 - 24 h before treatments, unless indicated otherwise. All cell treatments were done in absence of serum, in phenol red-free DMEM-F12 (Cellgro). Throughout all procedures, cells were regularly inspected by phase-contrast microscopy for changes in morphology.

NO donor treatments. Cells were seeded and grown as described above. The medium was replaced with phenol red-free DMEM-F12 containing the indicated concentrations of SIN-1, DETA/NO, Sper/NO or controls: SIN-1 vehicle (0.0001% HAc final), NONOate vehicle (0.1 mM NaOH final) or expired Sper/NO (left at 25°C, pH 7.4 for >24 h). DETA/NO has a half-life of 20 h at 37°C and neutral pH. Calculated according to Schmidt et al. (Schmidt et al., 1998), 1 mM DETA/NO will maintain a concentration of 2-3 µM NO in the incubation medium over at least 24
h. Sper/NO has a half-life of 39 min and 1 mM generates an initial flux of about $36 \mu M \cdot min^{-1}$ NO. Thus, in 1 mM Sper/NO media, the NO level peaks around 20 µM within 1 min, then gradually declines over 5 h (Schmidt et al., 1998). SIN-1 generates NO and superoxide in equimolar amounts resulting in a flux of roughly $30 \mu M \cdot min^{-1}$ ONOO$^-$ at 3 mM, that is maintained for at least 1 h (Ischiropoulos et al., 1995). NO formation by SIN-1 is undetectable unless superoxide dismutase is present. The release of ONOO$^-$ is much slower than its (auto)reaction rate (Pfeiffer and Mayer, 1998), so that ONOO$^-$ never reaches a steady-state concentration in media.

Typically, cells were treated for periods up to 24 h. In specified experiments, the NO donor-containing medium was removed after different periods of exposure, the cells were rinsed to remove all traces of NO donor and fresh serum- and phenol red-free DMEM-F12, with or without reagents, was added. Unless specified otherwise, reagent concentrations were 10 µM SB203580, 5 mM 3-aminobenzamide, 50 µM zVADfmk, 10 mg/l insulin (~1.7 µM), 3 ng/ml IGF-1 (~0.4 nM), 10 µM LY294002, 100 nM wortmannin and 0.1% (v/v) dimethylsulfoxide (DMSO). Incubations were then continued for a total period of 24 h. This method was applied to ensure results were from reagents acting on cells and not from non-specific interactions with NO donors in the medium.

**Assay of cell viability.** SH-SY5Y and NG108-15 cells were seeded and grown in 24-well plates as described above. After treatment(s) in phenol-red free DMEM-F12, the number of viable cells per well was determined using the *ATPLite-M* Cell Viability Assay from Packard (Downers Grove, IL) according to the manufacturer’s instructions. Briefly, medium was removed and cells were lysed *in situ*. Duplicate aliquots of lysate were transferred to an opaque 96-well plate. Substrate mix, containing luciferin and luciferase, was added and the ATP-dependent luminescence (cps/well) was measured in a Packard TopCount. In preliminary experiments, the ATP values were linearly correlated to the number of cells seeded per well. On the other hand,
total ATP also reflects the average ATP level of all the cells in the well. To assess whether ATP decreased not because cells were lost, but because cells lost ATP, we also determined the protein concentration in each well using remaining cell lysate (bicinchoninic acid (BCA) protein assay, Pierce, Rockford, IL).

**Griess nitrite assay.** Phenol-red free DMEM-F12 containing SIN-1 or Sper/NO was pipetted into a 24-well plate and placed in an incubator (37°C, 5% CO₂, >95% humidity) to mimic cell incubations conditions. At various time points, 180 µl medium was harvested and combined with 20 µl 0.1 mM HCl (SIN-1) or 1 mM NaOH (Sper/NO) on ice to stop the dissociation reaction. Levels of nitrite formed from the reaction of NO with H₂O, were determined using the Griess assay according to the manufacturer's instructions (Promega). Briefly, 50 µl medium sample or sodium nitrite standard was pipetted in duplicate in a 96-well plate. To each well, 50 µl 1% sulfanilamide in 5% phosphoric acid followed by 50 µl 0.1% N-1-naphylethylenediamine dihydrochloride, were added. Absorbance was read at 540 nm and nitrite concentrations calculated.

**Assay of cyclic GMP formation.** SH-SY5Y and NG108-15 cells were seeded and grown in 24-well plates as described above. The medium was aspirated and replaced by phenol red-free DMEM-F12 containing 0.25 mM phosphodiesterase inhibitor, IBMX (Sigma), in the absence or presence of 10 µM ODQ or ODQ vehicle (0.1% DMSO). After pre-incubation for 10 min at 37°C, various concentrations of DETA/NO were added. The reactions were terminated after 6 min with cold methanol and by placement in liquid nitrogen vapor for >1 h. Methanol was evaporated and the cell pellet extracted with 0.5 ml H₂O. Extracts were removed for determination of cyclic GMP by radioimmunoassay (Kreklau et al., 1999), whereas cell pellets were used for Bradford protein assay (BioRad Laboratories, Hercules, CA).

**Assay of caspase-3 activity.** SH-SY5Y cells were seeded and grown in 6-well plates as
described above. After treatment(s) in phenol-red free DMEM-F12, caspase-3 activity was determined using a colorimetric kit according to the manufacturer's instructions (R&D Systems). Briefly, medium was removed, cells were rinsed in phosphate-buffered saline (PBS, pH 7.6) and lysed in situ. Duplicate aliquots of lysate were transferred to a 96-well plate and substrate mix, containing Ac-DEVD-p-nitroaniline, was added. After incubation for 2 h at 37°C, absorbance was read at 405 nm and product concentration determined, using pure p-nitroaniline as standard. Remaining lysate was used to measure protein concentration (BCA, Pierce).

**DNA fragmentation analysis.** SH-SY5Y cells were seeded and grown in T25 flasks until approximately 80% confluent, and subjected to NO donor treatments in phenol red-free DMEM-F12, as described above. Suspended cells were collected with the medium and adherent cells were collected after trypsin/ethylenediamine-tetraacetic acid (EDTA) treatment. Cells were pelleted by centrifugation at 2,500 x g for 5 min, rinsed in PBS and lysed in 10 mM Tris, pH 8, 1 mM EDTA, 0.5% lauroyl sarcosine. Lysate was treated with 20 μg/ml RNase, for 10 min at 37°C, and with 250 μg/ml proteinase K, for 60 min at 55°C. DNA was precipitated in acid ethanol for 30 min at -20°C, and pelleted by centrifugation (16,000 x g for 15 min at 4°C). The pellet was rinsed with 70% ethanol and resuspended in 10 mM Tris, pH 8, 0.1 mM EDTA. Approximately one third of each DNA extract and 0.5 μg 1 kb DNA ladder (Promega) were loaded onto a 1.5% agarose gel containing 1 ng/ml ethidium bromide and DNA fragments were separated by electrophoresis at 95 V for 60 min. Bands were visualized under ultraviolet light and images were obtained using the Gel Doc 2000 gel documentation system (BioRad).

**SDS-PAGE and immunoblotting.** SH-SY5Y cells were seeded and grown for 18-24 h in 6-well plates, as described above. Cells were treated with NO donor or vehicle in phenol red-free DMEM-F12. After 5 h, NO donor medium was replaced with fresh DMEM-F12, with or without the following reagents. Cells were preincubated for 30 min in the absence or presence of 10 μM LY294002 whereupon 10 mg/l insulin or vehicle (0.0001% acetic acid) was added. After 10 min,
cells were harvested by scraping in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (50 mM Tris-Cl, pH 6.8, 4% 2-mercaptoethanol, 2% SDS, 20% glycerol). Proteins were separated by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (Hybond ECL, Amersham, Piscataway, NJ). Non-specific sites were blocked with 5% non-fat dry milk protein in Tris-buffered saline/0.1% Tween-20 (TBST) for 2 h at 25°C. After that, the membranes were probed with primary antibodies to phosphorylated protein kinase B (Akt), either rabbit phospho-Akt(Ser 473) or rabbit phospho-Akt(Thr 308) (Cell Signaling Technology, Beverly, MA), for 18 h at 4°C. After rinsing in TBST, the membranes were incubated with horse radish peroxidase-conjugated anti-rabbit IgG (Cell Signaling) for 1 h at 25°C. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL Plus; Amersham). To examine total Akt levels on the same membranes, antibodies were stripped off in 62.5 mM Tris, 2% SDS, 100 mM 2-mercaptoethanol for 30 min at 50°C. Membranes were rinsed 3x in TBST and reprobed as above except with rabbit Akt primary antibodies (Cell Signaling).

**Statistical analysis.** Data were statistically analyzed using Prism versions 3 and 4 (GraphPad Software Inc., San Diego, CA). Student’s t-test was applied for comparison of two groups. For multiple comparisons Analysis of Variance (ANOVA), followed by Bonferroni post-hoc test, was used. A probability of more than 95% ($p < 0.05$) was considered significant.

**RESULTS**

**Effects of NO donors on SH-SY5Y and NG108-15 cell viability**

To compare the effects of long-term exposure to NO and ONOO−, SH-SY5Y and NG108-15 cells were incubated for 24 h with various concentrations of DETA/NO, Sper/NO or SIN-1. All three treatments resulted in a dose-dependent decrease in cell viability (Fig. 1). NG108-15 cells were more resistant to the treatments than SH-SY5Y cells. However, as illustrated in Table I,
the differences in EC$_{50}$ between SH-SY5Y and NG108-15 cells were 15-fold for the NONOates, but only 2-fold for SIN-1. Moreover, in SH-SY5Y cells DETA/NO and Sper/NO were more potent than SIN-1, whereas this was reversed in NG108-15 cells. To examine whether the higher sensitivity to NONOates was specific for SH-SY5Y cells, we repeated the DETA/NO dose-response in another neuronal cell line, PC12. As shown in Table I, the EC$_{50}$ for DETA/NO in PC12 cells was not significantly different from that in SH-SY5Y cells. Based on these findings, we hypothesized that the mechanism whereby NONOates, i.e. NO, induced loss of cell viability, did not converge with that of SIN-1, i.e. ONOO$^-$. 

First, we examined whether the disparity was due to differences in activation of soluble guanylyl cyclase, a well-known NO target. As shown in Fig 2A, the potency of DETA/NO in stimulating cyclic GMP formation in SH-SY5Y and NG108-15 cells was similar, unlike that in causing loss of cell viability (Fig. 1A). Whereas this DETA/NO effect was inhibited by the soluble guanylyl cyclase inhibitor, ODQ (Fig. 2B), the effects on SH-SY5Y cell viability of neither DETA/NO, Sper/NO nor SIN-1 (0.3, 0.3 or 1 mM, respectively) were affected by ODQ (Fig. 2C). Similar effects of ODQ were seen in NG108-15 cells (data not shown). These findings excluded a role for cyclic GMP in the NO-induced loss of cell viability in SH-SY5Y and NG108-15 cells.

Another possibility we considered as cause for the higher NO sensitivity of SH-SY5Y and PC12 relative to NG108-15 cells, was the formation of nitrocatecholamines in these cell lines. To investigate this, we blocked dopamine synthesis in SH-SY5Y cells with the tyrosine hydroxylase inhibitor, $\alpha$-methyl-$p$-tyrosine. However, the presence of $\alpha$-methyl-$p$-tyrosine, in a range of concentrations, failed to inhibit DETA/NO-evoked loss of cell viability over 24 h (data not shown). Moreover, 48 h treatment with 0.1 mM $\alpha$-methyl-$p$-tyrosine itself decreased cell viability to 55.4 $\pm$ 7.5%, but did not affect the potency of DETA/NO (Fig. 3). Thus, the effects of DETA/NO and $\alpha$-methyl-$p$-tyrosine were mediated along different pathways.
Time-course of NO donor effects on cell viability

To get an estimate of the time-course of NO donor dissociation in our experimental conditions, we measured the formation of nitrite after incubation of SIN-1 in DMEM-F12. Roughly 30%, 0.89 of 3 mM SIN-1, decomposed to nitrite (Fig. 4, filled squares), consistent with data published for preformed ONOO⁻ (Pfeiffer and Mayer, 1998). Nitrite formation was linear with time for up to 1 h, with a slope of around 3.3 µM.min⁻¹ per mM SIN-1 (Fig. 4, dashed line). ONOO⁻ formation from 3 mM SIN-1 was projected to have an initial rate of 33 µM.min⁻¹ and to reach 2.3 mM over baseline (Fig. 4, open squares, dotted line). Nonlinear regression analysis gave an apparent half-life for SIN-1 of 26 min. This is much shorter than the reported 1 to 2 hours in serum and close to the 39 min half-life of Sper/NO (Fig. 4, open circles) (Keefer et al., 1996). The largest divergence in production of NO species between SIN-1 and Sper/NO was in the period between 1 and 2.5 hours. Of note, the time-course shown for Sper/NO is also theoretical rather than measured, because undissociated NONOates in the sample decompose instantly to nitrite in the acid conditions of the Griess assay.

To further characterize the different mechanisms of NONOates and SIN-1, we focused on SH-SY5Y cells. Our next step was to examine the effects of NO donors on SH-SY5Y cell viability over time. Cells were incubated with 1 mM DETA/NO, 1 mM Sper/NO or 3 mM SIN-1 - doses that resulted in approximately 80% cell death after 24 h (Fig. 1) - and cell viability was assayed after various shorter periods of incubation. As might be expected from its slow dissociation rate, DETA/NO had no significant effect during the first 5 h of incubation (Fig. 5A, triangles). Surprisingly, Sper/NO, which dissociates in a few hours, also did not affect cell viability until after 5 h of incubation (Fig. 5A, circles). Nevertheless, in the presence of SIN-1, a rapid decline to approximately 70% was observed at 30 min, followed by a temporary recovery and, after 5 h, a final decrease in cell viability (Fig. 5A, squares). Microscopic examination of the cells and protein determination revealed no changes in cell morphology or number during the
first 2.5 h, suggesting that SIN-1 induced a transient decline in cellular ATP levels. This biphasic effect was seemingly not related to the rate of SIN-1 dissociation, because Sper/NO did not acutely affect ATP levels (Fig. 5A, circles), even at a dose of 3 mM (not shown).

In a parallel set of experiments, cells were exposed to 1 mM DETA/NO, 1 mM Sper/NO or 3 mM SIN-1 for various periods of time as well, but incubation was then continued in NO donor-free medium and cell viability was measured after 24 h total. As shown in Fig. 5B, a brief exposure to 1 mM DETA/NO of up to 1 h, did not affect cell viability in the long term. Treatment with DETA/NO for 2.5 h or more resulted in a treatment time-dependent decrease in cell viability by 24 h (Fig. 5B, triangles). Again, Sper/NO exposure produced a similar gradual decline in cell viability (Fig. 5B, circles). Treatment with 1 mM Sper/NO for up to 5 h, during which 95% of the NONOate dissociated, still did not yield 80% loss of cell viability by 24 h, like full 24 h exposure did (Fig. 1C). This was not due to the removal of its breakdown product, spermine, because 24 h exposure to fully dissociated Sper/NO had no effect on cell viability (data not shown). At variance, 2.5 h exposure to 3 mM SIN-1 was sufficient to reach almost 80% loss of cell viability by 24 h (Fig. 5B, squares).

The inference from these results is that, despite the removal of NO donor before or at 2.5 h, when no effect on cell viability was noticeable (Fig. 5A), cell viability was nevertheless decreased after 24 h (Fig. 5B). Thus, short-term NO donor treatment initiated a process that led to loss of cell viability in the long term. A likely candidate for this process would be programmed cell death or apoptosis.

**Induction of apoptotic mechanisms in SH-SY5Y cells by NO donors**

To investigate whether NO donor-induced loss of cell viability involved apoptotic mechanisms, first, the effects of NO donors on DNA fragmentation were studied. SH-SY5Y cells were incubated overnight with 1 mM SIN-1, 0.3 mM SPER/NO or 1 mM DETA/NO, except that
DETA/NO was replaced by NO donor-free medium after 5 h. Under each of these conditions cell viability is decreased by approximately 50% (Figs. 1 and 5). As illustrated in Fig. 6, DNA from untreated cells (N) was mostly intact, as indicated by its electrophoretic mobility in the high molecular weight range. In DNA extracted from DETA/NO- (Fig. 6, left) and Sper/NO- (Fig. 6, right) treated cells, the characteristic ladder pattern resulting from internucleosomal cleavage by endonucleases was detectable. In contrast, DNA from SIN-1-treated cells migrated as a “smear” representing fragments of many, indeterminate sizes.

To further examine the involvement of apoptotic mechanisms, the effects of NO donors on caspase-3 activation were measured. SH-SY5Y as well as NG108-15 cells were treated with various doses of DETA/NO for 5 h and caspase-3 activity was assayed as described in Material and Methods. As shown in Fig. 7A, DETA/NO increased caspase-3 activation dose-dependently. A higher DETA/NO dose was needed to significantly increase caspase-3 activity in NG108-15 cells (Fig. 7A), in agreement with their higher resistance to DETA/NO-induced cell death (Fig. 1A). In addition, SH-SY5Y cells were treated with 1 mM DETA/NO, 1 mM Sper/NO or 3 mM SIN-1 - doses that resulted in approximately 80% cell death after 24 h (Fig. 1) - and caspase-3 activity was assayed after various periods of incubation. In SIN-1-treated cells, no change in caspase-3 activity was observed (Fig. 7B, squares), not even at a lower dose of 1.5 mM (data not shown). On the other hand, DETA/NO and Sper/NO both produced a significant 2- to 3-fold increase in caspase-3 activity by 5 h of incubation (Fig. 7B). In SH-SY5Y cells, DETA/NO-induced activity rose immediately and gradually, in accordance with its dissociation kinetics. Interestingly, Sper/NO treatment produced a steep increase in caspase-3 activity, but with several hours delay in view of its dissociation rate (Fig. 7B, open circles). Nevertheless, with the exception of SIN-1-treated cells, caspase-3 activation preceded the decrease in cell viability (compare Figs. 5A and 7).

Indeed, when data from Figs. 5B and 7B were plotted together, the caspase-3 activity in
DETA/NO-treated cells was inversely correlated with eventual cell survival (Fig. 8A). Considering the lack of caspase-3 activation in SIN-1 treated cells, there was no link with cell viability (Fig. 8C). In Sper/NO-treated cells an inverse relationship between caspase-3 activity and cell viability was not immediately obvious either (Fig. 8B). Only, when a 2.5 h time shift in the viability plot was applied, it could be seen that the steep decline in cell survival, induced by brief exposure to Sper/NO, mirrored a sheer rise in caspase-3 activity 19 to 21 h earlier (Fig. 8B, broken line). Taken together, these findings suggest that Sper/NO exposure gave a delayed response in caspase-3 activation as well as in loss of cell viability. Furthermore, the lack of both DNA laddering and caspase-3 activation after SIN-1-treatment, ruled out apoptosis as a mechanism of SIN-1-induced SH-SY5Y cell death.

Inhibition of NO donor-induced cell death in SH-SY5Y cells

Considering the delays between NO donor exposure, caspase-3 activation and cell death, we tested a variety of compounds for their ability to reverse the process: the poly(ADP) polymerase inhibitor 3-aminobenzamide, the pan-caspase inhibitor zVADfmk, and the p38 MAP kinase inhibitor SB203580. Cells were incubated with 0.3 mM Sper/NO or 1 mM SIN-1 for 5 h - treatments that had little short-term effect but resulted in approximately 50% cell death by 24 h. After 5 h, the medium was replaced with NO donor-free, inhibitor-containing DMEM-F12 and cell viability was measured after 24 h total. None of the above compounds was able to prevent cell death by NO donor treatment at generally effective doses (Fig. 9). A four-fold higher concentration (200 µM) of zVADfmk apparently enhanced cell survival, but this effect was found to be due to higher vehicle concentration (0.4% DMSO).

In addition, we considered whether activating cellular survival pathways, specifically those stimulated by insulin receptors, could prevent the loss of cell viability after NO donor treatment. To examine this, SH-SY5Y cells were again treated with NO donor for only 5 h. After
this, the medium was replaced with NO donor-free DMEM-F12 containing various concentrations of IGF-1 or insulin and cell viability was measured after 24 h total. Overnight incubation with IGF-1 significantly attenuated the loss of cell viability after 5 h treatment with 1 mM DETA/NO, in a dose-dependent manner with a maximum effect at 3 ng/ml (~0.4 nM) (Fig. 10A). Insulin also prevented loss of cell viability after 1 mM DETA/NO treatment, producing complete protection at 10 µg/ml (~270 U/l) (Fig. 10B). The increase in cell viability was blocked in the additional presence of PI3K inhibitor, either 10 µM LY294002 (Fig. 10A) or 100 nM wortmannin (Fig. 10B), indicating that both IGF-1 and insulin were acting through growth factor receptors. Insulin also enhanced cell survival after 5 h treatment with 3 mM DETA/NO or 0.3 mM Sper/NO, but was unable to protect cells from cell death induced by 1 mM SIN-1 (Fig. 11A). Moreover, 2 h of insulin post-treatment significantly inhibited caspase-3 activity in cells treated for 3 h with 1 or 3 mM DETA/NO or 1 mM Sper/NO, but had no effect in 3 mM SIN-1-treated cells (Fig. 11B). These results imply that activation of the growth factor receptor/PI3K pathway inhibited NONOate-induced apoptotic mechanisms in SH-SY5Y cells, but had no effect on the mechanism of SIN-1-induced cell death.

To further explore the insulin-stimulated growth factor receptor/PI3K survival mechanism, we examined the activation of PKB/Akt, a putative downstream effector in this pathway, by SDS-PAGE and Western blotting with phospho-specific antibodies. As shown in Fig. 12, A and B lower panels, 10 min of insulin treatment phosphorylated PKB/Akt at serine 473, but not at threonine 308. Nonetheless, increased pAkt(Ser473) levels did not correspond with increased cell viability in control and SIN-1-treated cells (Fig. 12A, first 4 lanes). Yet in Sper/NO-treated cells, PI3K inhibitor, 10 µM LY294002, inhibited insulin-stimulated cell survival as well as PKB/Akt phosphorylation (Fig. 12B). These findings suggest that Akt phosphorylation was required but not sufficient to mediate insulin-stimulated survival of SH-SY5Y cells.
NO donor effects in NG108-15 cells

Finally, we repeated some of the experiments in NG108-15 cells to test whether the difference between NO and ONOO\(^-\) mechanisms is unique for SH-SY5Y cells. In NG108-15 cells, 3 mM SIN-1, but not 3 mM Sper/NO, evoked a transient drop in NG108-15 cellular ATP levels (Fig. 13A). 5 mM SIN-1, unlike 5 mM DETA/NO, failed to increase caspase-3 activity (Fig. 13B). These are all doses that resulted in 80-100% loss of NG108-15 cell viability after 24 h (Fig. 1). NG108-15 cells did not respond to insulin or IGF-1. However, incubation in serum-containing media attenuated loss of NG108-15 cell viability, suggesting that other growth factors mediate cell survival. Thus, the differences between NO and ONOO\(^-\) effects on NG108-15 seem to parallel those in SH-SY5Y cells.

DISCUSSION

Nitric oxide (NO) contributes to cellular degeneration in various disorders. In combination with superoxide, NO forms the very reactive ONOO\(^-\) anion. ONOO\(^-\) is able to nitrate and oxidize protein residues, DNA and lipids, thereby affecting cellular homeostasis (Torreilles et al., 1999). It is a widely held assumption that the detrimental effects of NO on cell viability are mediated by ONOO\(^-\). Notwithstanding this view, several findings in the present study show that NO and ONOO\(^-\) have different mechanisms of inducing cell death. First, the NO donors, DETA/NO and Sper/NO, were 15-fold more potent in SH-SY5Y than in NG108-15 cells, whereas the EC\(_{50}\) of the ONOO\(^-\) donor, SIN-1, differed only 2-fold (Fig. 1, Table I). DETA/NO and Sper/NO induced a gradual loss of SH-SY5Y cell viability, while SIN-1 evoked a rapid, transient drop in cellular ATP prior to an abrupt decline in cell viability (Figs. 5A, 13A). Furthermore, DETA/NO and Sper/NO caused DNA laddering and caspase-3 activation, but SIN-1 treatment did not result in any signs of apoptosis (Figs. 6 and 7B). Finally, activation of the insulin receptor/PI3K/Akt cell survival pathway inhibited caspase-3 activation and prevented loss of cell viability in DETA/NO-
and Sper/NO-treated cells, but had no effect in SIN-1-treated cells (Figs. 10, 11 and 12). Taken
together, these data suggest that the detrimental effects of NO on cell viability do not involve
ONO\textsuperscript{−} formation.

This notion is also supported by other studies. In cardiac fibroblasts, ONOO\textsuperscript{−} scavengers
were ineffective in inhibiting DETA/NO- or cytokine-induced apoptosis (Tian et al., 2002). In rat
cerebral cortex neurons, brief ONOO\textsuperscript{−} pretreatment actually attenuated DETA/NO-induced
apoptosis (Garcia-Nogales et al., 2003). Trackey et al. (Trackey et al., 2001) showed that, in
mixed cortical cell culture, DETA/NO caused negligible nitrotyrosine formation, but had a greater
effect on cell viability as compared to SIN-1. Moreover, inhibition of ONOO\textsuperscript{−} formation by
inclusion of superoxide dismutase and catalase, enhanced cortical cell death, "suggesting that
the free NO formed from SIN-1 decomposition was slightly more neurotoxic than ONOO\textsuperscript{−} itself"
(Trackey et al., 2001).

Because also in our study pure NO and ONOO\textsuperscript{−} effects can be distinguished, differences
between the mechanisms of ONOO\textsuperscript{−}– and NO-induced cellular degeneration may be derived
from the characteristics presented here. As our ODQ experiments show, loss of cell viability was
not due to activation of soluble guanylyl cyclase by NO (Fig. 2, B and C). Preliminary
experiments have shown that SIN-1 indeed is able to evoke a rapid increase in cyclic GMP
production of ∼200-fold over basal (J.T.A. Meij, unpublished observations). This activation is
indirect through S-nitrosoglutathione intermediates (Schrammel et al., 1998). As well, the higher
sensitivity to NO of SH-SY5Y and PC-12 cells was not due to the presence of catecholamines.
Both \textit{in vitro} and \textit{in vivo}, catecholamine exposure to NO can yield 6-nitrocatecholamines and
these products have been implicated in NO neurotoxicity (d'Ischia and Costantini, 1995). On the
contrary, dopamine may well be an autocrine signal for cell survival, since treatment with α-
methyl-\textit{p}-tyrosine alone reduced cell viability (Fig. 3).

The time-courses of cellular degeneration give better clues to the different NO and
ONOO\(^{-}\) mechanisms. The effect of 3 mM SIN-1 was much faster than that of 1 mM Sper/NO (Figs. 5A and 13A), although both dissociated at comparable initial rates (Fig. 4) and gave similar results after 24 h. The acute effect of SIN-1 on ATP levels is consistent with many reports that ONOO\(^{-}\) inhibits mitochondrial respiration at complex I and/or complex III (Brookes et al., 2002; Almeida and Bolanos, 2001). This complex I block is irreversible, so what could be the basis of the temporary recovery seen in our experiments? It has been shown before that ONOO\(^{-}\)-dependent, transient depletion of mitochondrial ATP can cause delayed cell death (Almeida and Bolanos, 2001). In that study, the short-term recovery of ATP levels corresponded to a gradual decrease in NADPH levels and GSH/GSSG ratio. Thus, we speculate that in SIN-1-treated cells, intracellular redox mechanisms were also able to delay, but not avert, ATP depletion and cell death (Stamler et al., 2001). NO is known to affect mitochondrial respiration as well, except at the level of cytochrome c oxidase (complex IV) and this decreases \(O_2\) consumption without affecting ATP synthesis (Brookes et al., 2002). The acute effect of SIN-1, but not NONOates, on ATP levels is also consistent with the idea of differential effects of NO and ONOO\(^{-}\) on mitochondrial permeability transition and calcium handling, both processes that can contribute to cell death (Horn et al., 2002; Brookes and Darley-Usmar, 2004). Again, the effect of NO is thought to be reversible, whereas that of ONOO\(^{-}\) is not (Horn et al., 2002; Brookes and Darley-Usmar, 2004). Nevertheless, Clementi et al. (Clementi et al., 1998) have shown that persistent (> 6 h) exposure of cells to NO can eventually lead to mitochondrial inhibition, possibly through complex I S-nitrosylation, and ATP depletion. Thus, in contrast to rapid ONOO\(^{-}\) nitration, the NO effect on cell metabolism is late in onset and redox-reversible. The time courses and reversibility of the DETA/NO and Sper/NO effects (Figs. 5 and 11) certainly agree with such a process. However, if the main effect of insulin on cell viability was restoring redox balance, it probably could have prevented cell death in SIN-1-treated cells as well. Therefore, insulin may affect other cell processes.
The major insulin receptor/PI3K-mediated neuronal survival pathway involves phosphorylation of PKB/Akt at serine 473 (Datta et al., 1999). Activated PKB/Akt appears also involved in insulin-mediated survival of SH-SY5Y cells in our experiments (Figs. 9 and 12B). PKB/Akt is known to regulate both pro- and anti-apoptotic proteins. For instance, PKB/Akt suppresses the increase in Bad/Bcl-2 ratio, preventing mitochondrial leakage of cytochrome c (Datta et al., 1999; Banasiak et al., 2000). Furthermore, PKB/Akt can phosphorylate and, thereby, inhibit initiator caspase-9 (Datta et al., 1999; Cardone et al., 1998). Both actions block formation of the apoptosome - a pro-apoptotic complex consisting of cytochrome c, caspase-9, APAF-1 and ATP - and, hence, block activation of downstream death-promoting agents (Banasiak et al., 2000). Caspase-3 is one of those downstream effector enzymes that is blocked by the insulin receptor/PI3K/Akt pathway (Fig. 11B) (Banasiak et al., 2000). Because apoptosome formation requires ATP, caspase activation will only take place when ATP is available and, conversely, depletion of mitochondrial ATP levels leads to necrotic instead of apoptotic cell death (Brookes et al., 2002). Therefore, the opposite effects of NO and ONOO⁻ on (initial) ATP depletion may explain the differential activation of apoptotic mechanisms by NONOates and SIN-1, respectively (Figs. 6 and 7B). On the other hand, there was sufficient ATP left in SIN-1-treated cells to support phosphorylation of PKB/Akt (Fig. 12A).

NO and ONOO⁻ are also able to react with caspases (Mannick et al., 1999). ONOO⁻ mediated nitration of caspases could have contributed to the lack of SIN-1-induced caspase activation. If so, this inhibition obviously did not prevent cell death. Brief exposure to ONOO⁻ was sufficient to promptly reduce ATP levels (Fig. 5A, 13A) and later cause a major decline in cell viability (Fig. 5B). In addition, the dose-response curves of SIN-1-induced cell death were very steep (Figs. 1C) and we did not find any other signs of apoptosis in ONOO⁻ exposed cells. Indeed, the presence of a DNA “smear” instead of a “ladder” (Fig. 6, lane 5) is often associated with necrotic cell death. Therefore, we think that ONOO⁻ most likely caused cell collapse without
a cell death program.

S-nitrosylation of caspases by NO is reversible (Mannick et al., 1999). S-nitrosylation/denitrosylation might be responsible for the delay in caspase activation after Sper/NO treatment (Mannick et al., 1999). This scenario would imply that even before caspase activation, the cells were pre-programmed for apoptosis. Indeed, the data in Fig. 11B show increased caspase-3 activity, despite removal of Sper/NO 2 h earlier when activation was still absent (Fig. 7B). Also, we would have to assume that NO release by DETA/NO was too slow to inhibit caspases and that, in this respect, the effect of Sper/NO was a function of NO flux (Feelisch, 1998). Yet, the similar 24 h EC50 of DETA/NO and Sper/NO (Table I) suggest a near stoichiometric relationship between total NO released, irrespective of flux, and number of apoptotic cells. Furthermore, the virtually similar slopes in NG108-15 and SH-SY5Y cells (Fig. 1A,B) suggest a common mechanism for cell death past a certain cell type-specific NO threshold, possibly determined by levels of the proposed "NO sink" (Griffiths et al., 2002).

Can we extrapolate our findings in proliferating neuroblastoma cells to NO toxicity in differentiated cells in vivo? Their divergence in energy metabolism precludes extrapolation of the relative potencies and exact mechanisms of NO and ONOO− in vivo. The question our data, nevertheless, raise is whether the evidence for ONOO− as the sole damaging NO species in vivo is unequivocal or whether NO toxicity in vivo could be independent of ONOO− too? First, the presence of 3-nitrotyrosine is often taken to implicate ONOO− in cellular damage, but recent findings have given this notion considerable doubt (Pfeiffer et al., 2000; Espey et al., 2002). Reduced toxicity in animal models with enhanced superoxide scavenging is, in all likelihood, not due to reduction of ONOO− formation (Przedborski et al., 1992; Facchinetti et al., 1999). Further, as suggested by our data and demonstrated by Trackey et al. (Trackey et al., 2001), prevention of ONOO− formation only may not suffice because NO itself is equally or more toxic. In contrast, inhibition of NO synthesis (Aarts et al., 2002; Itzhak et al., 2000; Przedborski et al., 1996; Mabley
et al., 2004), blockade of apoptotic pathways (Mandir et al., 1999; Offen et al., 1998; Weisleder et al., 2004) and trophic supplements (Kordower et al., 2000; Gratton et al., 2001) are interventions that have been successful in animal models of NO toxicity. Therefore, it seems possible that NO rather than ONOO\(^-\) is the critical nitrogen species *initiating* cellular degeneration *in vivo*. Loss of trophic support may be key to its detrimental effects. Additional unfavorable conditions, such as modified calcium homeostasis, DNA damage, oxidative stress and the presence of inflammatory mediators or other toxic factors, may act to expedite and/or aggravate NO damage. Experiments to detect these factors should be done in differentiated and/or primary cells which may more closely resemble the *in vivo* situation. Our work lays a foundation for future identification of differential upstream mediators and co-factors of NO- and ONOO\(^-\)-induced cell death.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. NO donors have differential effects on SH-SY5Y and NG108-15 cell viability. SH-SY5Y (filled symbols) and NG108-15 cells (open symbols) were incubated in serum-free medium containing the indicated concentrations of A, DETA/NO, B, Sper/NO or C, SIN-1. After 24 h, the cell viability was determined using the ATPLite assay (Packard), as described under "Materials and Methods". Data represent percent of the values in absence of NO donor (mean ± SEM) from 4-5 experiments with duplicate determinations.

Fig. 2. Guanylyl cyclase inhibition does not prevent NO donor effects on cell viability. A, SH-SY5Y (filled triangles) and NG108-15 (open triangles) cells were incubated for 6 min at 37°C in serum-free medium containing IBMX and the indicated concentrations of DETA/NO. B, SH-SY5Y and NG108-15 cells were incubated for 6 min at 37°C in serum-free medium containing IBMX, 1 mM DETA/NO and either 10 µM ODQ (solid bars) or ODQ vehicle (0.1% DMSO, open bars). Cyclic GMP formation was determined as described under "Materials and Methods". Data represent percent of the values in absence of NO donor (mean ± SEM) from 3 experiments with triplicate determinations. Average basal values were 0.15 ± 0.03 and 10 ± 5 pmol/mg cyclic GMP in SH-SY5Y and NG108-15 cells, respectively (*p < 0.01, ODQ versus None). C, SH-SY5Y cells were incubated for 24 h in serum-free medium containing 0.3 mM DETA/NO, 0.3 mM Sper/NO or 1 mM SIN-1 and either 10 µM ODQ (solid bars) or ODQ vehicle (0.1% DMSO, open bars). Cell viability was determined as described under "Materials and Methods". Data represent percent of the values in absence of NO donor (mean ± SEM) from 4-5 experiments with duplicate determinations.

Fig. 3. Catecholamines are not involved in NO donor effects on cell viability. SH-SY5Y cells were pre-incubated for 24 h in growth medium containing None (filled triangles) or 0.1 mM α-
methyl-p-tyrosine (open triangles). Medium was then replaced with serum-free medium containing the earlier amount (None or 0.1 mM) of α-methyl-p-tyrosine and the indicated concentrations of DETA/NO. After incubation for another 24 h, cell viability was determined as described under "Materials and Methods". Data represent percent of the values in absence of DETA/NO and α-methyl-p-tyrosine (mean ± SEM) from 3 experiments with duplicate determinations.

**Fig. 4.** Time course of nitrite formation in SIN-1-containing medium shows rate of ONOO⁻ release. Serum-free medium containing 3 mM SIN-1 was incubated for the indicated times. Dissociation was terminated by the addition of ice-cold HCl. Levels of nitrite (filled squares) were determined using the Griess assay as described under "Materials and Methods". Data (mean ± SEM) are from 2 experiments with duplicate determinations. Dashed line represents linear regression analysis of values up to 1 h. Open squares are estimate of ONOO⁻ formed, whereas open circles indicate theoretical course of NO released by 1 mM Sper/NO.

**Fig. 5.** Time courses of NO donor effects on SH-SY5Y cell viability are different. A, SH-SY5Y cells were incubated in serum-free medium containing 1 mM DETA/NO, 1 mM Sper/NO or 3 mM SIN-1 for the indicated times. Cell viability was determined immediately, as described under "Materials and Methods". Data represent percent of the values in absence of NO donor (mean ± SEM) from 3-4 experiments with duplicate determinations. (*p < 0.05, **p < 0.001, SIN-1 versus control). B, SH-SY5Y cells were treated in serum-free medium containing 1 mM DETA/NO, 1 mM Sper/NO or 3 mM SIN-1 for the indicated times. Medium was then replaced with NO donor-free, serum-free medium and incubation continued until 24 h total. Cell viability was determined as described under "Materials and Methods". Data represent percent of the values in absence of NO donor (mean ± SEM) from 3-4 experiments with duplicate determinations.
determinations. (*p < 0.001, DETA/NO, Sper/NO, SIN-1 versus control)

**Fig. 6.** DNA fragmentation occurs after treatment with DETA/NO or Sper/NO, but not SIN-1. SH-SY5Y cells were incubated for 24 h in serum-free medium containing 1 mM DETA/NO (lanes 2, 3), 1 mM SIN-1 (lane 5), 0.3 mM Sper/NO (lanes 8, 9) or None (lanes 4, 7), whereby DETA/NO was replaced by NO donor-free medium after 5 h. Cells were harvested and DNA was extracted and separated as described under "Materials and Methods". Shown are representative results from 4 experiments. "L" indicates 1 kb DNA Ladder.

**Fig. 7.** Caspase-3 activity is increased after treatment with DETA/NO or Sper/NO, but not SIN-1. A, SH-SY5Y (solid bars) and NG108-15 (hatched bars) cells were incubated for 7 h in serum-free medium containing the indicated concentrations of DETA/NO. Caspase-3-like activity was determined as described under "Materials and Methods". Data represent percent of the values in absence of DETA/NO (mean + SEM) from 2 experiments with duplicate determinations. (*p < 0.05, **p < 0.001, DETA/NO versus untreated). B, SH-SY5Y cells were incubated for the indicated times in serum-free medium containing 1 mM DETA/NO (triangles), 1 mM Sper/NO (circles), 3 mM SIN-1 (squares) or vehicle. Caspase-3 like activity was determined as described under "Materials and Methods". Data represent percent of the values at the same time point in absence of NO donor (mean ± SEM) from 3-4 experiments with duplicate determinations. (*p < 0.001, Sper/NO or DETA/NO versus vehicle; #p < 0.01, Sper/NO versus DETA/NO).

**Fig. 8.** Caspase-3 activity can predict extent of loss of cell viability. The data for cell viability from Fig. 5B (filled symbols) and for caspase-3 activity from Fig. 7B (open symbols) for SH-SY5Y cells treated with A, 1 mM DETA/NO, B, 1 mM Sper/NO and C, 3 mM SIN-1 were plotted together. Broken line represents 2.5 h rightward shift of cell viability plot. Note symmetry
between cell viability and caspase-3 activity plots in A and B.

**Fig. 9.** Inhibitors of apoptosis do not rescue cells from NO donor treatment. SH-SY5Y cells were incubated in serum-free medium containing 0.3 mM Sper/NO, 1 mM SIN-1 or vehicle. After 5 h, medium was replaced with NO donor-free, serum-free medium containing vehicle (Ctrl), 10 µM SB203580 (SB), 50 µM zVADfmk or 5 mM 3-aminobenzamide (3-AB) and incubations were continued for an additional 19 h. Cell viability was determined as described under "Material and Methods". Data represent percent of the values in absence of NO donor and inhibitor (mean ± SEM) from 3-4 experiments with duplicate determinations.

**Fig. 10.** IGF-1 and insulin rescue cells after DETA/NO treatment. SH-SY5Y cells were incubated in serum-free medium containing 1 mM DETA/NO or vehicle. After 5 h, medium was replaced with NO donor-free, serum-free medium, A, containing 10 µM LY294002 (filled triangles) or vehicle (0.1% DMSO, open triangles), followed 20 min later by the indicated doses of IGF-1, or B, containing 100 nM wortmannin (filled triangles) or vehicle (0.1% DMSO, open triangles), followed 20 min later by the indicated doses of insulin. After 24 h total, cell viability was determined as described under "Materials and Methods". Data represent percent of the values in absence of NO donor (mean ± SEM) from 3 experiments with duplicate determinations. (*p < 0.05, **p < 0.01, IGF-1 or Insulin versus None; #p < 0.05, Wortmannin versus No inhibitor).

**Fig. 11.** Insulin protects SH-SY5Y cell viability and inhibits caspase-3 activation after treatment with DETA/NO or Sper/NO, but not SIN-1. A, SH-SY5Y cells were incubated in serum-free medium containing 3 mM or 1 mM DETA/NO, 0.3 mM Sper/NO, 1 mM SIN-1 or vehicle (not shown). After 5 h, medium was replaced with NO donor-free, serum-free medium containing None (open bars) or 10 mg/l insulin (solid bars). After 24 h total, cell viability was determined as
described under "Materials and Methods". Data represent percent of the values in absence of NO donor and insulin (mean ± SEM) from 3-5 experiments with duplicate determinations. (*p < 0.05, **p < 0.01, Insulin versus None). B, SH-SY5Y cells were incubated in serum-free medium containing 3 mM or 1 mM DETA/NO, 3 mM SIN-1, 1 mM Sper/NO or vehicle (not shown). After 3 h, medium was replaced with NO donor-free, serum-free medium containing None (open bars) or 10 mg/l insulin (solid bars). After 5 h total, caspase-3 activity was determined as described under "Materials and Methods". Data represent percent of the values in absence of NO donor and insulin (mean ± SEM) from 4 experiments with duplicate determinations. (*p < 0.05, **p < 0.01, Insulin versus None)

**Fig. 12.** PKB/Akt phosphorylation is involved in insulin-induced rescue of SH-SY5Y cell viability after NO donor treatment. A, SH-SY5Y cells were incubated in serum-free medium containing vehicle, 0.3 mM Sper/NO or 1 mM SIN-1. After 5 h, medium was replaced with NO donor-free, serum-free medium containing None (open bars) or 10 mg/l insulin (solid bars). Top panel: cells were harvested after 24 h total for determination of cell viability. Data are from experiments in Fig. 11. (**p < 0.001, Insulin versus None) Lower panels: cells were harvested 10 min after Insulin addition for SDS/PAGE-Western blotting with the indicated primary antibodies, as described under "Materials and Methods". B, SH-SY5Y cells were incubated in serum-free medium containing 0.3 mM Sper/NO or vehicle. After 5 h, medium was replaced with NO donor-free, serum-free medium containing 10 µM LY294002 (hatched bar) or vehicle (0.1 % DMSO) followed after 20 min by None (open bars) or 10 mg/l insulin (solid and hatched bars). Top panel: cells were harvested after 24 h total for determination of cell viability. Data represent percent of the values in absence of Sper/NO (mean ± SEM) from 3 experiments with duplicate determinations. (**p < 0.01, Insulin versus DMSO, Insulin+LY294002). Lower panels: cells were harvested 10 min after Insulin addition for SDS/PAGE-Western blotting with the indicated
primary antibodies, as described under "Materials and Methods".

**Fig. 13.** In NG108-15 cells, time course of SIN-1 effects on cell viability and caspase-3 activity are also different from those of DETA/NO and Sper/NO. A, NG108-15 cells were incubated for the indicated times in serum-free medium containing 3 mM Sper/NO, 3 mM SIN-1 or vehicle. Cell viability was determined as described under "Materials and Methods". Data represent percent of the values in absence of NO donor (mean ± SEM) from 3 experiments with duplicate determinations (**p < 0.01, SIN-1 versus control). B, NG108-15 cells were incubated for the indicated times in serum-free medium containing 5 mM DETA/NO, 5 mM SIN-1 or vehicle. Caspase-3 like activity was determined as described under "Materials and Methods". Data represent percent of the values in absence of NO donor (mean ± SEM) from 2 experiments with duplicate determinations. (*p < 0.05, **p < 0.01, Sper/NO versus control).
**Table 1.** EC50 values for NO donor-induced loss of cell viability in three neuronal cell lines.

Data are the best-fit values for logEC$_{50}$ ± SEM (DF=3-5), with the values for EC$_{50}$ (mM) in parentheses, from the experiments shown in Fig. 1. Note that the EC$_{50}$ for DETA/NO and Sper/NO are transformed to mark their dissociation into 2 mol NO per mol NONOate.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DETA/NO</th>
<th>Sper/NO</th>
<th>SIN-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>logEC$<em>{50}$ (EC$</em>{50}$)</td>
<td>logEC$<em>{50}$ (EC$</em>{50}$)</td>
<td>logEC$<em>{50}$ (EC$</em>{50}$)</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>-3.81 ± 0.04 (0.31)*</td>
<td>-3.72 ± 0.06 (0.38)*</td>
<td>-2.90 ± 0.07 (1.3)*†</td>
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<tr>
<td>NG108-15</td>
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<td>-2.59 ± 0.04 (5.1)</td>
<td>-2.53 ± 0.05 (2.9†)</td>
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<tr>
<td>PC12</td>
<td>-3.56 ± 0.12 (0.55)*</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* p<0.001 vs corresponding value in NG108-15 cells.

† p<0.001 vs other NO donors in same cell line.
Fig. 1

A. DETA/NO (log[M])

Cell viability (% of Control)

SH-SY5Y ▲ NG108-15

B. Sper/NO (log[M])

Cell viability (% of Control)

SH-SY5Y ● NG108-15

C. SIN-1 (log[M])

Cell viability (% of Control)

SH-SY5Y ■ NG108-15
Fig. 3

- **Cell viability (% of Control)**

- **DETA/NO (mM)**

- **No pretreatment**

- **α-methyl-β-tyrosine**
Fig. 4
Fig. 5

A. Cell viability (% of Control) vs Time (h)

B. Cell viability at 24 h vs Treatment period (h)

- DETA/NO
- Sper/NO
- SIN-1
Fig. 6
Fig. 7

A. SH-SY5Y and NG108-15 cells were treated with different concentrations of DETA/NO. The graph shows the percentage of Caspase-3 activity compared to control. The data points are indicated with asterisks: * indicates significance at p < 0.05, ** indicates significance at p < 0.01.

B. The graph shows the time course of Caspase-3 activity in response to DETA/NO, Sper/NO, and SIN-1 treatments. The symbols are: △ for DETA/NO, ○ for Sper/NO, and □ for SIN-1. The time points are indicated on the x-axis: 0.0, 2.5, 5.0, 7.5, and 24 hours.
Fig. 9
Fig. 10
Fig. 12

A. Cell viability (% of Control)

SIN-1
Sper/NO
Insulin

B. Cell viability (% of Control)

Sper/NO
Insulin
LY294002
DMSO
Fig. 13