Two distinct mechanisms of nitric oxide-mediated neuronal cell death show thiol dependency

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Gow, Andrew J., Qiping Chen, Madhura Gole, Marios Themistocleous, Virginia M.-Y. Lee, and Harry Ischirooulos. Two distinct mechanisms of nitric oxide-mediated neuronal cell death show thiol dependency. Am J Physiol Cell Physiol 278: C1099–C1107, 2000.—To better understand the mechanism(s) underlying nitric oxide (NO)-mediated toxicity, in the presence and absence of concomitant oxidant exposure, postmitotic terminally differentiated NT2N cells, which are incapable of producing ·NO, were exposed to PAPA-NONOate (PAPA/NO) and 3-morpholinosydnonimine (SIN-1). Exposure to SIN-1, which generated peroxynitrite in the range of 25–750 nM/min, produced a concentration- and time-dependent delayed cell death. In contrast, a critical threshold concentration (∼440 nM/min) was required for ·NO to produce significant cell injury. Examination of cells by electron microscopy shows a largely necrotic injury after peroxynitrite exposure but mainly apoptotic-like morphology after ·NO exposure. Cellular levels of reduced thiols correlated with cell death, and pretreatment with N-acetylcysteine (NAC) fully protected from cell death in either PAPA/NO or SIN-1 exposure. NAC given within the first 3 h posttreatment further delayed cell death and increased the intracellular thiol level in SIN-1 but not ·NO-exposed cells. Cell injury from ·NO was independent of cGMP, caspases, and superoxide or peroxynitrite formation. Overall, exposure of non-·NO-producing cells to ·NO or peroxynitrite results in delayed cell death, which, although occurring by different mechanisms, appears to be mediated by the loss of intracellular redox balance.

NITRIC OXIDE (·NO) has been implicated in cellular injury and death in neuronal degeneration (4). Most neuronal cells, especially those lacking the ability to generate ·NO, are vulnerable to damage by reactive nitrogen species and/or reactive oxygen species. These cells represent the majority of neurons, as only 1–2% of neuronal cells in the brain stain positively for neuronal ·NO synthase (13, 14, 27). Inhibition of ·NO synthesis has been shown to reduce neuronal injury mediated by glutamate, N-methyl-D-aspartate (NMDA) receptor activation, downregulation of superoxide dismutase, and trophic factor deprivation-induced motor neuron apoptosis (13, 16, 38). Further support for the role of ·NO in neuronal injury comes from animal models utilizing (NOS1) knockout mice. These mice were found to be resistant to stroke (15, 19), NMDA activation (3, 14), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity (32), and various mitochondrial neurotoxins (34).

There are a number of potential target molecules that may react directly with ·NO within the cellular environment, including heme-containing proteins, iron- and sulfur-containing proteins, and reduced thiols (27, 35). These reactions mainly are utilized in signal transduction and regulation of protein and are reversible. Mitochondria may be one of the major targets for ·NO since they are rich in iron-sulfur centers and iron-heme proteins. Although exposure to ·NO has been associated with cellular injury, it appears that endogenous superoxide production may be critical in mediating this injury (4, 15, 16, 23). The reaction of ·NO with superoxide leads to the formation of peroxynitrite, a higher redox potential oxidant and nitrating species. It has been suggested that the reactivity of peroxynitrite with proteins and mitochondria is irreversible and detrimental (9, 10, 23, 31). Indeed, protein tyrosine nitration, one of the peroxynitrite-induced protein modifications, is detected and localized to sites of neuronal injury induced by withdrawal of trophic support (16), mitochondrial toxins (34), MPTP (2), and NMDA activation (3). Therefore, the production of oxidants during exposure to ·NO could alter the biological targets affected by ·NO and hence the phenotypic outcome. Considering this complex interplay of variables, it is perhaps not surprising that ·NO has been implicated in both neuroprotective and neurodestructive mechanisms (27).

Reduced thiols may be critical reactants for ·NO and peroxynitrite (5, 8, 12, 33, 35, 39). We reasoned that reduced thiols, by controlling the intracellular concentration of these reactive species, could be critical in determining the phenotypic outcome after induction of nitrosative and/or oxidative stress. This study utilized postmitotic terminally differentiated NT2N cells as a model to study the cellular responses after exposure to kinetically defined fluxes of ·NO and/or peroxynitrite.

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MATERIALS AND METHODS

Assessment of nitric release rate in the culture medium. To examine the effects of nitrosative stress on neuronal cells in the presence and absence of oxidative stress, cells were exposed to chemical donor species PAPA-NONOate (PAPA/NO) and 3-morpholinosydnonimine (SIN-1; Calbiochem, La Jolla, CA). PAPA/NO, a member of the diazeniumdiolate · NO donors, is stable at alkaline pH and decays to release · NO at physiological pH (22). SIN-1, which is stable at acid pH, decays to release both · NO and superoxide at physiological pH (20). A number of factors have been shown to affect the rate of · NO release by donor species, including temperature, pH, presence of metal cations, serum components, and mixing rate (17, 18, 22). Therefore, to examine the effects of these donor species upon NT2N cells, it was essential to characterize the rate of release of · NO and/or peroxynitrite by these compounds in the NT2N culture medium. · NO release was determined by a · NO-selective electrode (ISO-NO; World Precision Instruments), as described in detail previously (17).

PAPA/NO rapidly established a steady-state release of · NO, reaching a plateau concentration of 470 nM that was maintained over a 2-h period. To maintain a steady-state level of 470 nM (assuming the rate of release equals the rate of removal), the rate of release from the PAPA/NO should approximate 220 nM/min. This is based on the experimental determination of the rate of decay of 470 nM authentic · NO in the NT2N culture medium. Addition of superoxide dismutase did not increase the release of · NO, indicating that superoxide was not formed in the culture media during decomposition of PAPA/NO. Consistent with previous studies, addition of SIN-1 to NT2N culture medium produced minimal release of · NO, indicating that the culture medium does not contain electron acceptors, which have been shown to be important for converting SIN-1 to a · NO donor (17, 18). However, when sufficient superoxide dismutase was added to the medium to remove superoxide, a steady-state level of 820 nM of · NO release is observed from 1 µM SIN-1. The effect of superoxide dismutase on the observed release of · NO is indicative that, under normal conditions, the · NO released by SIN-1A is captured by the superoxide generated by the reduction of oxygen by the electron released during ring opening of SIN-1. Production of peroxynitrite from the decomposition of SIN-1 was confirmed by the oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine. Previous data indicated that DHR 123 is oxidized by peroxynitrite and not by either superoxide, · NO, or hydrogen peroxide alone (20). The yield of rhodamine formation after a 2-h incubation in the NT2N media at 37°C was measured from the absorbance at 500 nm ($\varepsilon_{500 \text{nm}} = 78,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$). Therefore, by adjusting the initial concentration of the donor compound (PAPA/NO, SIN-1), the cells were exposed to kinetically defined fluxes of · NO or peroxynitrite.

Cell culture and exposure of cells to · NO and peroxynitrite. NT2N cells were cultured as described previously (11). Briefly, the cells were plated at a density $\sim 1.0 \times 10^6$ cells/well in six-well 35-mm tissue culture plates coated with polylysine. Cells were grown in macrophage-conditioned DMEM with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin for 2 wk. The culture contained nearly mature neuronal phenotype demonstrated by their axons and dendrites (Fig. 1). To maintain uniformity, the same passage of cells was used throughout these experiments. Solutions of PAPA/NO and SIN-1 were prepared as 100 mM stock in 0.1 N NaOH and phosphate buffer, pH 5, respectively, and were stored in nitrogen-purged air-tight bottles in the dark at $\sim 20°C$. The concentration of the stock solution of PAPA/NO was determined by measuring the absorbance at 250 nm ($\varepsilon_{250 \text{nm}} = 8,050 \text{ M}^{-1} \cdot \text{cm}^{-1}$) before use. The cells were rinsed twice with PBS to remove nonadhered cells, 2 ml of fresh medium containing PAPA/NO or SIN-1 was added, and cells were incubated at 37°C for 2 h. Cells were exposed to air every 15 min with a gentle rocking to avoid SIN-1 decomposition-induced hypoxia. In a static solution, the decomposition of SIN-1 will consume oxygen and form superoxide. Because the concentration of oxygen dis-
solved in biological media is ~220 µM, the cells can be made hypoxic if aeration is not performed. In addition, if oxygen is depleted, the decomposition of SIN-1 will not form peroxynitrite. At the end of incubation, cells were washed extensively and incubated in fresh medium for various periods.

Determination of cell viability. Rhodamine 123 (R123) and YO-PRO1 (Molecular Probes, Eugene, OR) fluorescent dye were used to determine the cell viability, as described previously (18). Rhodamine 123 was loaded at the final concentration of 10 µM 1 h before exposure to PAPA/NO or SIN-1. YO-PRO1 (2 µM) was added at the same time as the donors. An additional 2 µM of YO-PRO1 was added after cells were washed, and the cells were kept in the medium until the end of incubation. Rhodamine 123 is a negatively charged amphipathic molecule used as a marker of the cellular ability to maintain intracellular charged compartments (21). YO-PRO1, a DNA-binding and membrane-impermeant dye, is used as a marker of the loss of plasma membrane integrity. Cell viability was determined by counting the number of cells with rhodamine 123 or YO-PRO1 fluorescence under an inverted microscope (Olympus IX 70). Three images obtained with a ×20 optical lens from each well were used to count YO-PRO1 cellular fluorescence. Cellular injury was determined as follows: %cells YO-PRO1 labeled = (no. of cells YO-PRO1 labeled/no. of cells seen in phase contrast) × 100.

Cellular concentration of reduced thiol determination. The cells were washed and harvested in 200 µl of buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 4 mM EGTA. An aliquot was used to quantify both protein and nonprotein thiols using a thiol quantification kit (Molecular Probes) according to directions provided by the manufacturer. An aliquot of the cell lysate was used for DC-protein assay (Bio-Rad). The total reduced thiol concentration was normalized to cellular protein.

Electrolyte microscopy. The cells were washed with 0.1 M sodium cacodylate buffer (pH 7.3) 18 h after exposure and fixed in the plates for 1 h at 4°C with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. At the end of fixation, cells were washed two times with ice-cold 0.1 M sodium cacodylate buffer and harvested gently with a rubber scraper. The cell were washed two times with ice-cold 0.1 M sodium cacodylate buffer. The fixed cells were dehydrated in graded acetone and infiltrated with graded Polybed 812 epoxy resin. The samples were embedded in a polybed and polymerized at 60°C for 48 h overnight. Other experimental procedures. For restoration of reduced thiol levels, cells were treated with 10 mM N-acetylcysteine (NAC; Aldrich, Milwaukee, WI) 1 h before exposure or at different times postexposure to PAPA/NO and SIN-1. To examine the possible effect of caspases in PAPA/NO- and SIN-1-induced cell death, YVAD (50 µM), an inhibitor of caspase 1, and DEVD (50 µM), an inhibitor of caspase-3 (Calbiochem), were preincubated with cells before PAPA/NO and SIN-1 exposure. The role of cGMP- and cGMP-dependent pathways on PAPA/NO- and SIN-1-initiated cell injury was evaluated by addition of 1) cell-permeable cGMP analogs 100 µM 8-bromo-cGMP and/or 200 µM 8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP; Calbiochem), 2) guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (10 µM), and 3) cGMP-dependent protein kinase inhibitor Rp diastereomer of 8-pCPT-cGMP (100 µM; all from Calbiochem).

Statistics. Results are reported as means ± SD unless otherwise stated. Statistical significance was determined by one-way ANOVA with individual comparisons by Dunnett’s post hoc test. The level of statistical significance was taken as P < 0.05.

RESULTS

Cell injury as a function of flux and length of exposure. Figure 1 represents a typical experiment of exposure of NT2N cells to NO donor and SIN-1 using fluorescent dyes to assess cell viability. Cellular injury was found to be dependent upon the magnitude of exposure (Fig. 2A). However, it appears that exposure to PAPA/NO results in minimal cellular injury until a threshold exposure between 220 and 440 nM/min·NO is reached. In contrast, there is a gradual increase in toxicity with exposure to SIN-1. Furthermore, the degree of cellular injury resulting from exposure is also dependent upon the length of time of the exposure (Fig. 2B). Exposure to either of the decomposed donor compounds did not result in cell injury (not shown). Moreover, the loss of rhodamine 123 fluorescence in NO- and SIN-1-treated cells indicates depolarization of the mitochondrial membrane potential. Rhodamine 123 fluorescence decreased with exposure to SIN-1 even at the lower fluences and returned to control levels after a 1-h washoff period. The Rhodamine 123 fluorescence was found to be dependent upon the magnitude of exposure (Fig. 2A). However, it appears that exposure to PAPA/NO results in minimal cellular injury until a threshold exposure between 220 and 440 nM/min·NO is reached. In contrast, there is a gradual increase in toxicity with exposure to SIN-1. Furthermore, the degree of cellular injury resulting from exposure is also dependent upon the length of time of the exposure (Fig. 2B). Exposure to either of the decomposed donor compounds did not result in cell injury (not shown). Moreover, the loss of rhodamine 123 fluorescence in NO- and SIN-1-treated cells indicates depolarization of the mitochondrial membrane potential. Rhodamine 123 fluorescence decreased with exposure to SIN-1 even at the lower fluences and returned to control levels after a 1-h washoff period.
accumulates in the charged membrane compartments of living cells, and the loss of fluorescence indicates membrane depolarization (18, 21).

Time course of cellular injury and levels of reduced thiol. The time course of the appearance of YO-PRO1 labeling and hence injury is shown in Fig. 3A. There is no sign of injury up to 3 h postexposure, but there is a steady increase in the number of YO-PRO1-labeled cells from 5 to 18 h. Approximately 50% of the cells are YO-PRO1 labeled by 9 h. After 18 h, there is little increase in cell death under these conditions. Under conditions in which PAPA/NO and SIN-1 exposure induced the same degree of injury, there is little variation in the time course.

Data in Fig. 3B reveal a temporal association between reduced thiol content and cellular injury. Reduced thiol content is expressed as a percent of the control level normalized to protein content. Therefore, this reduction is not merely a loss of thiol as a result of the loss of cellular material. It is a reduction in the intracellular concentration of reduced thiol, since the intracellular protein concentration remains constant. This temporal association does not establish whether a reduction in intracellular thiol content is the cause or effect in these cellular injury processes. However, treatment of NT2N cells with buthione sulfoximine (which leads to a reduction in glutathione levels) for 2 h also resulted in a delayed cellular injury (data not shown).

NAC protection of cell injury. The decrease in the intracellular thiol concentration suggests that maintenance of redox potential either during the exposure period or in the chase period might provide some protection against injury. Figure 4A shows a temporal association between reduced thiol content and cellular injury. The decrease in the intracellular thiol concentration during exposure and at 1 and 3 h postexposure (time points at which there is no visible cellular injury). Posttreatment with NAC did not affect the cellular injury response to NO; however, at all three time points, there was a significant reduction in cellular injury in response to SIN-1 exposure at 18 h (Fig. 4B).

Having established that pretreatment with a reduced thiol provided protection against NO-related cellular injury, we examined whether cells could be rescued in the period postexposure to a toxic flux of either NO or peroxynitrite. NAC was added immediately after exposure and at 1 and 3 h postexposure (time points at which there is no visible cellular injury). Posttreatment with NAC did not affect the cellular injury response to NO; however, at all three time points, there was a significant reduction in cellular injury in response to SIN-1 exposure at 18 h (Fig. 4B). Examination of cell viability at 24 h showed that the degree of cell death had not changed with NAC posttreatment, i.e., that maximal cell death was now being reached at 24 rather than 18 h. This delay of the cell death process was reflected in the maintenance of the intracellular reduced thiol content at control levels in these cells (Fig. 4C). NAC posttreatment did not increase intracellular reduced thiol concentration in NO-treated cells.

Morphological and biochemical examination of cell death. The mechanism of cell death was investigated by the use of caspase 1 and 3 inhibitors. Inhibition of caspase 1 and 3 did not rescue cells from exposure to either PAPA/NO or SIN-1 (Table 1), and NT2N lysates did not have any measurable caspase activity. Figure 5 depicts representative images of cells examined by electron microscopy 18 h after exposure. The majority of cells treated with NO are in various stages of an apparently apoptotic cell death process as evident by the appearance of swollen bodies, condensation of the nuclear material, and fragmentation (Fig. 5). A small percentage (10%) of the cells showed evidence of necrosis after NO exposure, whereas exposure to peroxynitrite revealed only necrotic cells (Fig. 5).

Cellular injury was not mediated by cGMP, as it could not be reproduced by the addition of the cGMP analogs 8-bromo-cGMP and 8-pCPT-cGMP (Table 2). Cell injury was also independent of guanylate cyclase and cGMP-dependent kinases since cell injury was not blocked by inhibition of either guanylate cyclase or cGMP-dependent protein kinase (Table 2).
Cell injury after exposure to PAPA/NO was not prevented by preincubation with the superoxide and peroxynitrite scavenger manganese tetrakis(4-benzoyl acid)porphyrin (MnTBAP) as 90.9 ± 4.9% (n = 3) of cells were labeled with YO-PRO 18 h after exposure. MnTBAP was used at a concentration (150 µM) that has been shown previously to protect cells from ·NO-mediated death (16). Thus it appears that the ·NO-induced cell death is independent of endogenous superoxide and peroxynitrite formation.

**DISCUSSION**

This study confirms and extends previous observations regarding the role of ·NO and peroxynitrite in cell homeostasis and injury. Exposure of NT2N cells to a
flux of either \( \cdot \text{NO} \) or peroxynitrite results in delayed cellular injury. There is no immediate sign of injury during the exposure period, and several hours after the exposure, the ability of the cell to maintain the charge potential of intracellular compartments and the plasma membrane integrity is only compromised. This is consistent with the work of Burney et al. (8) who reported induction of delayed cell death upon exposure of other cell types to \( \cdot \text{NO} \) under kinetically defined conditions. However, the cellular injury processes induced by expo-

Table 1. Effect of caspase inhibitors

<table>
<thead>
<tr>
<th></th>
<th>YVAD</th>
<th>DEVD</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.7 ± 0.9</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td>PAPA/NO</td>
<td>87.6 ± 5.7</td>
<td>92.6 ± 2.0</td>
</tr>
<tr>
<td>SIN-1</td>
<td>93.7 ± 2.0</td>
<td>92.0 ± 2.3</td>
</tr>
</tbody>
</table>

Values represent means ± SD of % cells labeled with YO-PRO 18 h after exposure from 3 independent experiments. Cells were treated with YVAD (50 µM), an inhibitor of caspase 1, and DEVD (50 µM), an inhibitor of caspase 3, 1 h before, during, and 2 h after exposure to PAPA-NONOate (PAPA/NO) and 3-morpholinosydnonimine (SIN-1).

Fig. 5. Morphological examination of cell death. Cell exposed to SIN-1 demonstrated the absence of cellular membrane but normal nuclear appearance. A-D: cells exposed to PAPA/NO displaying vacuolar degeneration and different stages of apoptotic-like morphology and chromatin condensation.
Table 2. Effect of cCMP- and cGMP-dependent pathways in cell death after PAPA/NO exposure

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PAPA/NO</th>
</tr>
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<tbody>
<tr>
<td>No addition</td>
<td>3.2 ± 0.6</td>
<td>88.4 ± 1.7</td>
</tr>
<tr>
<td>8-Bromo-cGMP</td>
<td>3.8 ± 0.4</td>
<td>92.8 ± 3.9</td>
</tr>
<tr>
<td>8-pCPT-cGMP</td>
<td>4.2 ± 2.4</td>
<td>88.3 ± 4.1</td>
</tr>
<tr>
<td>ODQ</td>
<td>2.6 ± 1.2</td>
<td>92.3 ± 2.2</td>
</tr>
<tr>
<td>Rp-8-pCPT-cGMP</td>
<td>3.7 ± 1.1</td>
<td>90.7 ± 0.5</td>
</tr>
</tbody>
</table>

Values represent the means ± SD of % cells labeled with YO-PRO-1 18 h after exposure from a duplicate determination of 2 independent experiments. The cells were treated with cell-permeable cGMP analogs, 100 µM 8-bromo-cGMP and 200 µM 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP), as well as with a guanylyl cyclase inhibitor, 10 µM 1H-[1,2,4]oxadiazolo[4,3-c]quinoxalin-1-one (ODQ), or 100 µM of the Rp diastereoisomer of 8-pCPT-cGMPs (Rp-8-pCPT-cGMPs), a cGMP-dependent protein kinase inhibitor, immediately after a 2-h exposure to buffer (Control) or PAPA/NO.

...sure to PAPA/NO or SIN-1 appear to be different. First, the dose-response curves for the two agents appear to differ (Fig. 2A). Second, NAC treatment in the first 3 h postperoxynitrite exposure further delayed cell death but had no effect on ·NO exposure. Third, and most significantly, there are clear morphological differences by electron microscopy, with ·NO exposure producing a mostly apoptotic morphology, whereas SIN-1 exposure resulted in a mostly necrotic appearance. Finally, cell injury from ·NO exposure appears to be independent of intracellular superoxide and peroxynitrite formation, as pretreatment with superoxide and/or peroxynitrite scavengers did not prevent the injury. Pretreatment of cells with MnTBAP, a superoxide and peroxynitrite scavenger that has been shown to rescue motor neurons from apoptosis induced by trophic factor deprivation (16), did not provide protection. Moreover, it was confirmed that superoxide was not generated in the NT2N cell media during decomposition of the ·NO donor and, taken together with the lack of protection by MnTBAP, indicates that the ·NO-induced injury is independent of superoxide and peroxynitrite formation. These observations highlight the specificity of cellular responses to these forms of stress. However, other cell types have been shown to respond differently to ·NO and peroxynitrite. Exposure to low fluxes of peroxynitrite induced apoptotic death in cerebrocortical cells cultured from embryonic rats, in rat motor neurons, and HL-60 cells (6, 16, 26), whereas exposure of oligodentrocytes to relatively high fluxes of ·NO resulted in necrosis (29). The chemical nature of the reactive species may be important in determining the outcome in some cell types, as peroxynitrite has been shown to activate the cleavage of procaspase 3 to active caspase (26), and ·NO has been shown to inhibit the activation and function of caspase 3 (24, 28). Overall, the chemical nature of the reactive species, its concentration, duration of exposure, and cell type may determine the type of cell death.

Although the cellular responses to ·NO and peroxynitrite appear to be different, there are some strong similarities. Unlike other cell types (24, 26, 30), cell injury from ·NO and peroxynitrite was not dependent upon either cGMP or caspase 1 and 3 (Tables 1 and 2). Cell injury from both species resulted in the loss of reduced intracellular thiol and a temporal association between this reduction and the loss of cell viability. Cellular injury in response to either treatment can be abrogated by pretreatment with NAC. Addition of NAC even 3 h after exposure to SIN-1 augmented the intracellular levels of reduced thiols and delayed cell death. These results imply that SIN-1-mediated injury may occur via a loss of reducing equivalents that leads to initiation of cell death, and posttreatment with NAC maintains the reducing environment and thus delays cell death. However, it is interesting to note that, although NAC pretreatment raised the postexposure level of reduced thiol, it did not return it to control levels. Therefore, the relationship between reduced thiol concentration and degree of cellular injury is not the sole determinant of exposure effect, indicating that thiol treatment does not merely maintain the reducing equivalents in the cell. Indeed, the ability of NAC to inhibit ·NO-mediated injury only when used as a pretreatment indicates that it may be acting as a sacrificial target. Moreover, the dose-response curve for PAPA/NO exposure suggests that cellular injury becomes apparent once the flux of ·NO is high enough to overcome an intracellular barrier. It is possible that this barrier is the reduced thiol content of the cell.

A potential target for both ·NO and peroxynitrite within the cell is the mitochondrion. Indeed, a number of mitochondrial enzymes have been shown to be inhibited by ·NO, including complexes I and IV, cytochrome oxidase, and aconitase, resulting in a decrease in energy production (9, 10, 12, 18, 31). The morphological changes, cellular swelling, and vacuolar degeneration, as well as the loss of rhodamine 123 fluorescence that occurred in most cells exposed to ·NO resembled other models of mitochondrial dysfunction-driven cell death (18, 34, 41). Peroxynitrite also has been shown to inactivate aconitase and complexes I and II (9, 10). Moreover, within neuronal cells, the intracellular level of glutathione, and hence reduced thiol, has been shown to be critical in determining the effectiveness of ·NO inhibition of mitochondrial function (5, 12). The inhibition of mitochondrial respiration and the collapse of mitochondrial membrane potential has been associated with significant reductions in cellular redox capacity, energy depletion, and potentially death (5, 7, 39, 40). Energy depletion and activation of poly(ADP) synthase have been previously shown to play a critical role in ·NO/ peroxynitrite-mediated cytotoxicity (37).

Moreover, mitochondrial function appears to be critical in determining the mode of cell death in response to glutamate exposure (1), and mitochondrial-derived apoptotic stimuli are capable of inducing cell death independently of caspases (36).

The experiments shown here demonstrate that a neuronal cell line, NT2N, is sensitive to ·NO exposure both in the presence and absence of a concomitant oxidative stress. It is possible that the sensitivity of these cells to ·NO exposure is derived by the lack of an adaptive response, since these cells are not capable of producing ·NO and thus do not have the opportunity to develop a response and adapt to nitrosative stress. It remains to be seen whether low levels of ·NO exposure...
can lead to an adaptive response and hence resistance. Furthermore, the sensitivity of NT2N to NO and peroxynitrite is in part mediated by intracellular reduced thiols. These data provide potential mechanisms for NO-mediated cellular injury and highlight the importance of intracellular reduced thiols, and hence the redox state of the cell in general, in resistance to nitrosative stress.

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REFERENCES

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