Characterization of the Cytoprotective Action of Peroxynitrite Decomposition Catalysts

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The formation of the powerful oxidant peroxynitrite (PN) from the reaction of superoxide anion with nitric oxide has been shown to be a kinetically favored reaction contributing to cellular injury and death at sites of tissue inflammation. The PN molecule is highly reactive causing lipid peroxidation as well as nitration of both free and protein-bound tyrosine. We present evidence for the pharmacological manipulation of PN with decomposition catalysts capable of converting it to nitrate. In target cells challenged with exogenously added synthetic PN, a series of metalloporphyrin catalysts (5,10,15,20-tetrakis(2,4,6-trimethyl-3,3-disulfonatophenyl)porphyrinato iron (III) (FeTMPS); 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) (FeTPPS); 5,10,15,20-tetrakis(N-methyl-4'-pyridyl)porphyrinato iron (III) (FeTMPyP)) provided protection against PN-mediated injury with EC50 values for each compound 30–50-fold below the final concentration of PN added. Cytoprotection was correlated with a reduction in the level of measurable nitrotyrosine. In addition, we found our catalysts to be cytoprotective against endogenously generated PN in endotoxin-stimulated cells as well as in dissociated cultures of hippocampal neurons and glia that had been exposed to cytokines. Our studies thus provide compelling evidence for the involvement of peroxynitrite in cytokine-mediated cellular injury and suggest the therapeutic potential of PN decomposition catalysts in reducing cellular damage at sites of inflammation.

The excess production of nitric oxide, generated primarily by the inducible nitric-oxide synthase (iNOS), has been implicated as a mediator of cellular injury at sites of inflammation (1, 2). However, recent evidence supports a role for peroxynitrite (PN) in the cellular damage and death (3, 4) once attributed entirely to nitric oxide (NO). The formation of this powerful oxidant from nitric oxide and superoxide anion, free radical species frequently generated by activated infiltrating leukocytes, is essentially diffusion-limited. Peroxynitrite has been shown to cause lipid peroxidation (5), chemical cleavage of DNA (6, 7), inactivation of key metabolic enzymes such as aconitate (8, 9), ribonucleotide reductase, succinate dehydrogenase, and cytochrome oxidase of the mitochondrial electron transport chain (10, 11), and reduction in cellular antioxidant defenses by oxidation of thiol pools (12). PN can also nitrate protein tyrosine residues, possibly leading to inactivation of tyrosine kinase activity (13) or to the disruption of key cytoskeletal components that may contribute to the pathogenesis of diseases such as amyotrophic lateral sclerosis (14) or ALS. At physiologic or acidic pH, the protonated form of PN is a short lived molecule. As a result of this instability, the detection of nitrotyrosine has become a reliable biochemical marker for the presence of peroxynitrite in pathophysiological processes. Nitrotyrosine has been detected in tissues from Alzheimer’s (15), multiple sclerosis (16), ALS (14, 17), and rheumatoid arthritis (18) patients, suggesting a role for peroxynitrite in the pathogenesis of these diseases. It should be noted, however, that evidence for PN-independent routes of nitrotyrosine formation have been recently demonstrated (19), indicating that careful consideration should be taken when assigning the source of oxidative damage in a disease process.

There is accumulating evidence arising from different in vitro cellular systems that the generation of PN occurs during cytokine stimulation leading to cellular injury and death (20–23). Both apoptotic and necrotic pathways for cell death have been invoked as consequences of exposure to PN (24–27). In this report, we test the hypothesis that by pharmacologically manipulating the effective concentration of either exogenously added or endogenously produced PN, we can protect cells from injury. The peroxynitrite decomposition catalysts FeTMPS, FeTPPS and FeTMPyP (28, 29) were cytoprotective in both experimental paradigms, demonstrating that the catalytic shunting of PN to an innocuous form, i.e. nitrate, may have therapeutic utility in reducing tissue damage during inflammation.

EXPERIMENTAL PROCEDURES

Cell Culture—RAW 264.7 cells and the human adenocarcinoma cell line, DLD-1, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 5% CO2 and 37 °C. Dissociated cultures of rat hippocampal neurons were grown on a glial feeder layer as described previously (30, 31) with the following modifications. Briefly, 1-day old Sprague-Dawley (Charles River, MA) rat pups were sacrificed, and their hippocampi were surgically removed and then dissociated both mechanically and enzymatically by papain digestion. Debris was removed by low speed centrifugation. Cells were plated at a density of 50,000 cells per well on a 96-well plate containing a confluent layer of glia (initial plating of approximately 3100 cells per well 5–6 days previously). Cultures were grown for 7 days in MEM (minus phenol red) supplemented with 0.5% glucose, 0.22% NaHCO3, 2

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1 The abbreviations used are: iNOS, inducible nitric-oxide synthase; FeTMPS, 5,10,15,20-tetrakis(2,4,6-trimethyl-3,3-disulfonatophenyl)porphyrinato iron (III); FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III); FeTMPyP, 5,10,15,20-tetrakis(N-methyl-4'-pyridyl)porphyrinato iron (III); PN, peroxynitrite; NO, nitric oxide; ALS, amyotrophic lateral sclerosis; l-NMA, L-nitro-arginine-methyl ester; l-NIL, L-nitro-arginine-l-lysine; SOD, superoxide dismutase; SNP, sodium nitroprusside; BSA, bovine serum albumin; LPS, lipopolysaccharide.
mm l-glutamine, and 10% NU-Serum I (Becton Dickinson). No antimitic agents were used. A mixture of cytokines was then added to induce iNOS in this culture system: lipopolysaccharide (0111:B4) at 100 to 200 ng/ml; interferon γ (200 to 400 units/ml); tumor necrosis factor α (150 to 300 units/ml); interleukin 1β (5–10 ng/ml). Five to seven days following the addition of cytokines, the cultures were analyzed for nitrite production and cell viability.

**Rat Aortic Ring Preparation and Assay for the Interaction of NO with PN Catalysts**—Four aortic rings were prepared from each of five rats. Briefly, thoracic aortas were removed from adult Sprague-Dawley rats anesthetized by injection with 10 mg/kg xylazine and 50 mg/kg ketamine. Connective tissue was carefully trimmed to avoid damage to the endothelium. Rings were cut into 3-mm lengths and placed into a 10 ml tissue bath. In some experiments, the endothelium was removed by gentle rubbing of the ring preparation. Successful removal of the endothelium was confirmed by the lack of a relaxation response to acetylcholine (10 µM). Aortic rings were maintained at 37 °C in Krebs bicarbonate buffer, pH 7.2. 130 mM NaCl, 15 mM NaHCO3, 5 mM KCl, 1.2 mM Na2HPO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM d-glucose, and 0.1 mM ascorbic acid bubbled with 5% CO2, 95% O2. Rings were preloaded with 1 g of tension and equilibrated for 30 min with 2–3 buffer changes. After stabilization of the base line, the rings were contracted with 0.1 µM phenylephrine, producing 90–100% contraction. Nitrergic oxide-mediated relaxation of the precontracted, endothelium-intact rings was produced by the addition to the buffer of NO gas in the concentration of approximately 10 mM acetylcholine (1 mM to 10 µM) or by treatment of endothelium-denuded rings with the NO donor, sodium nitroprusside (1 mM to 10 µM). Test compounds were added to the tissue bath 10 min prior to acetylcholine or sodium nitroprusside addition. Isometric tension was recorded, and relaxation was determined as the percentage of maximum tone developed to phenylephrine.

**PN-mediated Tyrosine Nitration**—Peroxynitrite and PN decomposition catalysts (28, 29) were synthesized as described previously. The PN concentration was determined prior to use by measuring the absorbance at 302 nm and using an extinction coefficient of 1.67 mM−1 cm−1. Bovine serum albumin (BSA) was dissolved at a concentration of 5 mg/ml in 100 mM potassium phosphate buffer, pH 7.4, that had been vigorously degassed with nitrogen. A 2 mM NO solution was prepared by several minutes of bubbling NO gas into the same buffer at 37 °C and 5% CO2 to assess cellular viability. At the end of the assay, the solution was treated with 30 µg/ml of radioiodinated donkey anti-rabbit IgG (protein concentration of approximately 10 µg/ml, Amersham Pharmacia Biotech). After further washing to remove unbound secondary antibody, the blot was exposed for 5 min or more on a PhosphorImager (Molecular Dynamics). PN-treated BSA was used as the nitrotyrosine standard to determine the level of protein-bound nitrotyrosine. The specific enrichment of this enzyme, the levels of which were taken into account by measuring its presence in the conditioned medium of untreated controls.

**Immunocytochemical Staining**—Immunocytochemical staining was performed as described previously (34) with the following modifications. Primary cultures of neuronal cells and glia (predominantly astrocytes) were treated with cytokines as described above for 5–7 days. To assess viability, representative fields of phase bright cells with neuronal morphology and excluding trypan blue were counted in each of four separate wells. In most cases, a radioimmunoassay for neuron-specific enolase (NSE) was also performed to confirm the cell counts since the value obtained for the Alamar Blue assay was a better indicator of glial viability than of neuronal health (glia were present in larger numbers than neurons by the end of each experiment).

**Neuron-specific Enolase Quantitation**—In order to assess neuronal viability, release of neuron-specific enolase (NSE) from injured and dying neurons into the culture medium was quantitated by radioimmunoassay as described by the manufacturer, Amersham Pharmacia Biotech. Briefly, 150 µl of conditioned medium was assayed for the γ isoyme of neuron-specific enolase, which, unlike lactate dehydrogenase, is not present in glia. However, serum containing a small amount of this enzyme, the levels of which were taken into account by measuring its presence in the conditioned medium of untreated controls.

**Measurement of Nitrite and Nitrate**—Nitrite concentrations were measured as described previously using a fluorometric assay (35). When indicated, nitrate was converted to nitrite by nitrate reductase before measurement. Nitrate was converted to nitrite by nitrate reductase before measurement. Nitrate reductase was added to the reaction mixture prior to the addition of 0.2% phenylmethylsulfonyl fluoride (PMSF) to inhibit nitrate reductase activity and to add 10 µg/ml of PMS. Nitrate reductase activity was measured by monitoring the decrease in absorbance at 220 nm resulting from the reduction of the NADP to NADPH. The reaction was started by the addition of 0.1 M substrate. The reaction was monitored for 3 min, and the rate of increase in absorbance was determined.

**Results**

**Reduction in Protein Nitrotyrosine Content As a Measure of PN Decomposition Catalyst Activity**—To assess the activity of compounds as PN decomposition catalysts, FeTMAPS was tested for its ability to reduce the level of protein nitrotyrosine content of bovine serum albumin that had been treated with synthetic peroxynitrite. Fig. 1A shows a Western blot for nitrotyrosine content of BSA treated with either 2 mM nitric oxide (lione 1) or...
1 mM peroxynitrite (lane 4) in the presence (lane 5) or absence of PN catalyst in PBS. As expected, the nitrotyrosine content of PN-treated BSA (lane 4) was greatly elevated over BSA alone (lane 7), NO-treated BSA (lane 1), or PN allowed to decompose in PBS for 15 min prior to addition (lane 8). The nitrotyrosine content of the PN-treated BSA was decreased in the presence of the active catalyst, FeTMPS (10 μM, lane 5), while TMPS (an inactive compound) failed to reduce the detectable signal (lane 6). Antiserum specificity for nitrotyrosine was demonstrated either by competition with excess nitrotyrosine (10 mM) or by conversion of nitrotyrosine to aminotyrosine with 1 mM dithionite (data not shown). Fig. 1B shows the results using a direct radioimmunoassay for nitrotyrosine to analyze the same samples that had been spotted onto nitrocellulose. This quantitative measure for nitrotyrosine confirmed the qualitative results using the Western analysis and was not limited to only protein that had entered the gel. It is interesting to note that the free ligand TMPS, lacking an iron center, produced an apparent increase in the measurable levels of nitrotyrosine, a result that may be explained by the observation that trace contamination with metals such as iron, especially in the presence of potential chelating agents such as TMPS, can catalyze PN-mediated nitration of tyrosine (36).

Fig. 2 illustrates the concentration-dependent reduction of the nitrotyrosine content of BSA treated with PN (1 mM) in the presence of increasing amounts of the active catalyst FeTMPS. This contrasted with the profile obtained using ascorbate, a known PN scavenger, where concentrations equimolar (or higher) to the PN challenge were required to see an effect. TMPS, on the other hand, did not reduce the level of protein-bound nitrotyrosine, indicating that it is neither a PN catalyst nor a scavenger of PN (data not shown).

**Immunocytochemical Detection of Nitrotyrosine on Cells Treated with PN**

To investigate the ability of PN catalysts to reduce the PN-mediated modification of proteins in a cellular milieu, immunohistochemical staining of cells in culture for nitrotyrosine was performed. The human adenocarcinoma cell line DLD-1 showed surprising resistance to PN-mediated cellular damage (data not shown). When cells were treated with increasing concentrations of PN, a corresponding increase in the number of cells that stained with nitrotyrosine-specific rabbit antisera (Fig. 3A) was observed. When the final concentration of PN was kept constant at 200 μM, cell staining was decreased in a concentration-dependent manner using the active catalyst FeTMPS (Fig. 3B). All positive staining was competed by incubation with excess nitrotyrosine (data not shown).

**PN Decomposition Catalysts Protect Cells from PN-mediated Injury**

Cellular injury is a very real consequence of exposure to PN, a powerful oxidant of not only protein but also nucleic acid and lipid. A typical concentration-response curve for cellular injury to RAW 264.7 cells by exogenously added peroxynitrite is shown in Fig. 4A. Cellular viability was measured by monitoring the reduction of the electron acceptor Alamar Blue to a fluorescent product by the mitochondrial electron transport chain. Fig. 4B and C, illustrates the cytoprotection afforded by FeTMPyP and FeTPPS, respectively, against exogenously added synthetic PN.

Nitrotyrosine is a relatively specific and stable biochemical marker for the generation of PN. The mechanism of action of PN decomposition catalysts should favor a reduction in the nitrotyrosine content of cellular proteins exposed to PN. When the level of protein-bound nitrotyrosine following a PN pulse was measured by radioimmunoassay analysis of cellular contents either released into the medium (Fig. 5A) or remaining on cells (data not shown), there was a strong correlation between nitrotyrosine level and the size of the PN pulse (Fig. 5A). In both cases, the level of nitrotyrosine was reduced in a concentration-dependent manner by the active compound FeTMPyP (Fig. 5B).

Fig. 6 illustrates the difference in the concentration-response
profile for a PN catalyst compared with a typical free radical scavenger such as ascorbate which reacts stoichiometrically with the oxidant until its reactive moieties are depleted. There was an essentially complete protection of RAW cells from a 200 μM PN challenge in the presence of 10 μM FeTMPS. This is in sharp contrast to ascorbic acid which was fully protective at a 2-fold molar excess to PN, and to TMPS which was inactive at all concentrations tested. Nitrotyrosine content of released intracellular proteins was used as a marker for the extent of PN-mediated injury in these cells. FeTMPS reduced the level of detectable protein-bound nitrotyrosine release (Fig. 6B).

There was no cytoprotection observed when FeCl₃ was used or when catalyst was added after PN application (data not shown). The free ligands TPPS, TMPyP, and TMPS are inactive as decomposition catalysts and were unable to protect from PN-mediated damage.

Cytokine-mediated Injury Is Attenuated by Treatment with PN Decomposition Catalysts—We next examined the cytoprotective capacity of PN decomposition catalysts against endogenously produced PN. The murine monocyte-macrophage cell line, RAW 264.7, can be stimulated to express iNOS with endotoxin. Enzyme activity was monitored by measuring nitrite accumulation in the conditioned medium. As shown in Fig. 7B, the induction of iNOS markedly reduced cell viability (≈20%). Inhibition of cellular iNOS activity with l-NMA or the selective iNOS inhibitors aminoguanidine (data not shown) and l-NIL (32, 39, 40) resulted in a concentration-dependent increase in cellular viability accompanied by a corresponding decrease in accumulated nitrite (Fig. 7) when compared with the endotoxin control.

As shown in Fig. 8, co-incubation of the PN decomposition catalysts with endotoxin increased cellular viability when examined 24 h after the beginning of treatment. Fig. 8 illustrates the cytoprotective profile for the active catalysts FeTMPS (Fig. 8A) and FeTPPS (Fig. 8B). The free ligands (i.e. TMPS, TPPS) afforded cytoprotection at much higher concentrations than the active catalysts. To rule out metabolic conversion of the free ligand to an active form, the inactive analog ZnTMPS and the weak catalyst MnTPPS were tested (Fig. 8B). Neither compound showed protective effects at the highest concentrations applied.

We next extended our studies utilizing cell lines to primary cultures of neurons and glia, cells known to be susceptible to cytokine-mediated injury. In fact, cytokine-mediated injury accompanied by expression of iNOS and cyclooxygenase-2 may contribute significantly to the neural damage associated with human disorders such as stroke. Accordingly, we examined the cytoprotection afforded by PN decomposition catalysts on mixed cultures of dissociated hippocampal neurons and glia (predominantly astrocytes) treated with a mixture of cytokines and endotoxin. Cellular viability was assessed using several
biochemical criteria: for neurons, by counting phase bright trypan blue impermeant cells exhibiting neuronal morphology and by measuring neuron-specific enolase release from injured and dying cells; and for glia (comprising the majority of the cells per well), by measuring the release of lactate dehydrogenase from injured and dying cells or by assessing the cellular capacity to convert Alamar Blue to its fluorescent product by functional mitochondria. Primary cultures were exposed to cytokines and endotoxin for 5–7 days at the end of which viability and NOS activity were quantitated. Fig. 9 summarizes the results that we obtained using FeTMPS. Although there was typically no concentration-dependent decrease in accumulated nitrite and nitrate by the cells (data not shown), there was a profound protection of both neurons and glia by treatment with either FeTMPS (Fig. 9) or FeTMPyP (data not shown) when compared with the inactive analogue, ZnTMPS. The pharmacological response profile differed between neurons (Fig. 9, A and B) and glia (Fig. 9C), with glial cells being more amenable to cytoprotection. Nevertheless, PN catalyst protection was evident in either case. The only obvious difference was morphological, with glial cells maintaining their morphology while neurons largely withdrew their processes from the glial feeder layer at the higher concentrations of catalyst used (10 and 20 μM). This result was in sharp contrast to L-NIL treatment which preserved neuronal morphology with a coincident drop in nitrite production (data not shown) and neurototoxicity (Fig. 9).

Lack of Interaction between NO and PN Catalysts—To rule out the catalytic removal of NO by the PN catalysts as a possible explanation for their cytoprotective action, we examined their effect on guanylate cyclase-mediated relaxation of rat aortic rings. The effect of FeTMPs and FeTMPyP on endothelium-dependent (endogenously produced NO) and -independent (exogenously added NO) relaxation in rat aortic rings was examined. Acetylcholine-mediated relaxations are dependent upon the release of nitric oxide from the endothelium while its removal eliminates the vasorelaxant effect of this vasodilator (1). Fig. 10A demonstrates that acetylcholine (1–100 nM) -mediated relaxation of phenylephrine (0.1 μM) -preconstricted aortic rings was not affected by preincubation with either FeTMPS or FeTMPyP (300 μM). In contrast, hemoglobin (5 μM), which is known to bind to and completely inactivate NO, abolished the acetylcholine-mediated relaxation. To further illustrate that acetylcholine was producing its effects by stimulating NO generation, the NOS inhibitor L-NAME (100 μM) was added to the organ bath. Like hemoglobin, L-NAME completely inhibited the relaxations. The relaxation of precontracted endothelium-denuded aortic rings following the exogenous addition of sodium nitroprusside (SNP), an NO donor, at concentrations ranging from 1 nM to 1 μM was also unaffected by the presence of FeTMPS or FeTMPyP (300 μM). In contrast to the catalysts, hemoglobin (5 μM) eliminated the relaxation response of the denuded rings (Fig. 10B). Thus, regardless of the source of NO, PN catalysts produced no detectable inhibition of the guanylate cyclase-mediated relaxation of vascular smooth muscle. Conversely, because SOD and SOD-mimics clearly potentiate the activation of guanylate cyclase by NO (1, 37), the lack of a PN catalyst-mediated...
increase in the NO-driven relaxation response of aortic rings suggests that any SOD mimetic activity associated with the catalysts is negligible.

**DISCUSSION**

A greater understanding of the role played by peroxynitrite in the pathogenesis of human disease will aid in the design of rational therapies for pharmacological intervention. Peroxynitrite is thought to possess a dual free radical nature capable of hydroxyl radical-like lipid peroxidation and NO$_2$-driven nitration of tyrosine. Stability of PN is pH-dependent, with protonation resulting in either decomposition into predominantly nitrate or the initiation of oxidative processes including lipid peroxidation and the nitration of tyrosine. Thus, a compound capable of diverting PN into a “non-destructive” pathway would increase nitrate while reducing free radical-initiated damage to critical molecular targets within the cell. The chemical synthesis and proposed mechanism of action of such compounds have recently been described (28, 38) as has their efficacy in in vivo models of inflammation (29). The purpose of this study was to more fully characterize the cytoprotective properties of this novel class of compounds, the peroxynitrite decomposition catalysts.

Inherent in the transient nature of many free radical species is the difficulty in measuring their presence in more complex living systems. While lipid peroxidation can result from Fenton/Haber-Weiss chemistry, tyrosine nitration represents a stable biochemical marker, which with a few noteworthy exceptions (19), is relatively specific for peroxynitrite generation (4). We have presented evidence for a reduction in protein nitrotyrosine content when synthetic PN is added to BSA in the presence of the active catalyst FeTMPS; the inactive compound TMPS, which lacks the iron center of active catalysts, did not reduce PN-mediated tyrosine nitration, indicating that it is neither a catalyst nor a scavenger of PN. To assess the activity of the catalysts on a broader array of protein targets within a cellular context, human adenocarcinoma cells (DLD-1) treated with PN showed positive immunostaining for nitrotyrosine. As was observed with BSA, PN decomposition catalysts caused a concentration-dependent reduction in PN-generated nitrotyrosine staining of cellular protein.

Because the physiological consequences of peroxynitrite generation are associated with tissue damage, we next tested the utility of peroxynitrite decomposition catalysts in preventing PN-mediated cellular injury and death. Peroxynitrite, the product of the diffusion-limited reaction of superoxide anion with nitric oxide, has been shown to participate in both apoptotic and necrotic cellular injury (24–26). Diseases in which PN has been implicated include ischemia-reperfusion injury,
stroke, Alzheimer’s disease, ALS, multiple sclerosis, and rheumatoid arthritis (14–18). In fact, there is compelling evidence for PN generation by cytokine and LPS-treated cells. It includes analysis of predicted nitrite/nitrate ratios (20, 41, 42) as well as the direct measurement of nitrotyrosine formation by HPLC/mass spectroscopy (43), the detection of catalase-insensitive but superoxide dismutase/NOS inhibitor-sensitive 1,2,3-rhodamine formation (21), and immunohistochemical staining of primary cultures of cytokine-stimulated glia (22).

We have utilized the monocyte-macrophage line, RAW 264.7, both as a target for exogenously added PN and as a cellular source of endogenously produced PN. To determine the efficacy of PN catalysts, we initially used a defined pulse of synthetic PN in our studies. FeTMPS, FeTPPS, and FeTMPyP were cytoprotective at concentrations 30–50 fold below the size (final concentration) of the PN challenge. The inactive (free ligand) analogues did not protect. This protection depended on the prior presence of catalyst and was not apparent with FeCl3 alone. Perhaps most compelling was the difference between concentration-response (cytoprotection) curves for FeTMPS and ascorbate. Ascorbate followed a scavenger activity profile necessitating a 2-fold molar excess of the anti-oxidant over the size of the PN pulse in order to be totally effective. This requirement for a molar excess of free radical scavenger (or reactive groups contained within such an antioxidant) is consistent with that observed for uric acid and cysteine when exposed to PN (44, 45). PN-generated nitrotyrosine formation in cellular proteins was also reduced in a concentration-dependent manner by PN catalyst, a result comparing favorably with its EC50 value for cytoprotection (approximately 5 μM for FeTPPS).

Endotoxin and cytokines have been used to induce iNOS in our studies. FeTMPS, FeTPPS, and FeTMPyP were cytoprotective at concentrations 30–50 fold below the size (final concentration) of the PN challenge. The inactive (free ligand) analogues did not protect. This protection depended on the prior presence of catalyst and was not apparent with FeCl3 alone. Perhaps most compelling was the difference between concentration-response (cytoprotection) curves for FeTMPS and ascorbate. Ascorbate followed a scavenger activity profile necessitating a 2-fold molar excess of the anti-oxidant over the size of the PN pulse in order to be totally effective. This requirement for a molar excess of free radical scavenger (or reactive groups contained within such an antioxidant) is consistent with that observed for uric acid and cysteine when exposed to PN (44, 45). PN-generated nitrotyrosine formation in cellular proteins was also reduced in a concentration-dependent manner by PN catalyst, a result comparing favorably with its EC50 value for cytoprotection (approximately 5 μM for FeTPPS).
treatment, suggesting NO-driven mitochondrial dysfunction, a well documented effect on mitochondrial electron transport (10, 46). Treatment of these cells with catalyst also resulted in cytotoxicity by this criterion, an observation consistent with peroxynitrite-mediated cell injury. Uninduced cells incubated overnight with catalyst did not show any significant loss in viability (data not shown), although compound seemed to be taken up into cells as evidenced by the visible reddish-brown tint of the cell layer after washing.

Neurons of the central nervous system, especially those of the hippocampus, are very susceptible to cytokine-mediated injury especially in the context of the later stages of stroke (47–52). Glial cells, on the other hand, seem to be more resistant to such injury (11). In vitro generation of PN by cytokine-activated glia has recently been demonstrated (22). Primary cultures of rat hippocampal neurons and glia subjected to cytokine insult for 5–7 days of treatment with cytokines. This staining correlated well with the expression of iNOS (22). We decided to test the cytoprotective capacity of PN decomposition catalysts on primary cultures of rat hippocampal neurons and glia. We found the astrocytic feeder layer more amenable to protection by these compounds when compared with their neuronal counterparts. Accumulated levels of nitrite were usually unaffected, suggesting that NOS inhibition is not part of the mechanism of action of PN decomposition catalysts.

In summary, our data demonstrate the utility of PN decomposition catalysts as cytoprotective agents against PN-mediated cellular injury and death. Our data are also consistent with their proposed chemical mechanism of action by revealing a measurable reduction in protein nitrosyline content of PN-treated cells in culture. Moreover, the preservation of cellular viability from PN-mediated damage is critical to any consideration of the potential therapeutic value of PN catalysts. Where peroxynitrite fits into the pathogenesis of diseases such as Alzheimer’s and multiple sclerosis and whether it is a causative agent or merely a marker for a pathological faite accompli remain very much open questions. Answers to questions such as these will define the clinical utility of drugs capable of intercepting PN and catalyzing its conversion into innocuous metabolites such as nitrate.

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