Nitric oxide (NO) is a free radical that is endogenously produced by the enzyme NO synthase (NOS), which catalyzes the oxidation of L-arginine, yielding NO and L-citrulline \((1,2)\). NO regulates various cellular functions via cyclic GMP-dependent and -independent mechanisms \((3,4)\), and these effects are critical in the physiological regulation of nervous, immune, and vascular systems. It is important to note, however, that excessive or inappropriate formation of NO might cause deleterious effects relevant in various human pathologies such as acute endotoxicemia, neurological disorders, atherosclerosis, and ischemia/reperfusion \((3,5)\). Although NO can be directly detrimental to cells that produce large amounts of NO after stimulation may have some resistance mechanisms to the toxic effects mediated by peroxynitrite \((4,42,43)\). These discrepancies are a possible consequence of differences in the peroxynitrite concentrations used and/or mode of peroxynitrite administration \((e.g.,\) as a precursor or as a bolus). Additional factors that might affect the lethal response evoked by peroxynitrite are the composition and the pH of the solutions in which the cells are treated. Indeed, although specific components of the extracellular milieu can interact with peroxynitrite, changes in the pH from physiological to alkaline values can increase the half-life of the oxidant, thus prolonging its activity toward target cells \((44,45)\). Several studies have used treatment conditions at pH values ranging between 8.6 and 9 \((32,37,40)\). Finally, an important factor to consider is the cell type. Astrocytes were reported to be more resistant than neurons to the toxic effects mediated by peroxynitrite \((5,10)\), and it is generally believed that cells that produce large amounts of NO after stimulation may have some resistance mechanism against their own peroxynitrite. Thus, it appears that the toxic response and mode of response does not appear to be directly triggered by peroxynitrite but rather seems to be mediated by reactive oxygen species generated in the respiratory chain, most likely at the level of complex III. Additional studies revealed that superoxide dismutase mimetic agents suppressed both the release of arachidonic acid and the oxidation of a superoxide-sensitive fluorescent probe mediated by peroxynitrite. Because under the same conditions the oxidation of a hydrogen peroxide-sensitive fluorescent probe was unchanged, it appears that superoxides play a pivotal role in peroxynitrite-dependent activation of PLA2. These results therefore suggest that downstream products of the PLA2 pathway may play a role in the lethal response evoked by peroxynitrite.
cell death mediated by peroxynitrite vary in different cell types and under different treatment conditions.

We recently reported experimental evidence consistent with the notion that increasing concentrations of peroxynitrite fail to induce apoptosis in U937 cells (46). A proportion of these cells, however, were found to die by necrosis via a mitochondrial permeability transition–dependent mechanism. This response, and the ensuing cell lysis, was extremely rapid, and the cells that survived this treatment did not undergo delayed apoptosis (or necrosis) but rather proliferated with kinetics superimposable on those observed in untreated cells. Thus, an all-or-nothing mechanism appears to regulate the fate of U937 cells challenged with peroxynitrite: some cells undergo an extremely fast necrotic response, whereas the remaining cells are fully viable and capable of performing energy-demanding functions such as proliferation.

Similar results were obtained in recent studies from our laboratory using PC12 cells exposed to a short-chain lipid hydroperoxide analog, tert-butyl hydroperoxide. Under these conditions, endogenous peroxynitrite was found to mediate various effects, including DNA single-strand breakage (47). Cell death induced by the hydroperoxide also appeared to be mediated by peroxynitrite because it was markedly reduced by NOS inhibitors as well as by NO and peroxynitrite scavengers (48). Furthermore, morphological and biochemical analyses revealed that the mode of cell death was necrosis and that this response was causally linked to peroxidation of membrane lipids and mitochondrial permeability transition (48).

Direct versus Indirect Effects of Peroxynitrite

Peroxynitrite is a highly reactive species and is commonly thought to interact with, and damage, various biomolecules. It is also well established that peroxynitrite is extremely short-lived at physiological pH values, and the formation of 3-nitrotyrosine by peroxynitrite reaction with tyrosyl residues is often used as a stable marker. An additional approach to indirectly measure peroxynitrite formation involves the use of the fluorescent probe dihydrorhodamine 123 (DHR), which accumulates in mitochondria when oxidized by various reactive species, including peroxynitrite. The ability of peroxynitrite to oxidize DHR is very well established, and inhibition of the DHR fluorescence response by NOS inhibitors or NO or peroxynitrite scavengers is commonly interpreted as a clearcut indication of peroxynitrite formation. We recently reported (49), however, that this was not the case in PC12 cells treated with either exogenous peroxynitrite or tert-butyl hydroperoxide, an agent resulting in the formation of endogenous peroxynitrite, as described above. Under these conditions, DHR was not directly oxidized by peroxynitrite; rather, this response appeared to be mediated by peroxynitrite-dependent activation of PLA₂. The following lines of evidence supported this inference: a) the DHR fluorescence response elicited by tert-butyl hydroperoxide was blunted by low concentrations of two structurally unrelated PLA₂ inhibitors; b) low levels of authentic peroxynitrite restored the DHR fluorescence response in NOS-inhibited cells but not in PLA₂-inhibited cells, whereas reagent arachidonic acid was effective under both conditions; c) the DHR fluorescence response induced by authentic peroxynitrite was also blunted by PLA₂ inhibitors and restored upon addition of reagent arachidonic acid. We therefore conclude that endogenous, or exogenous, peroxynitrite does not directly oxidize DHR in intact cells. Rather, peroxynitrite appears to act as a signaling molecule promoting release of arachidonic acid, which in turn leads to formation of species causing oxidation of DHR.

Thus, a messenger function of peroxynitrite may not be responsible only for DHR oxidation because it can be expected that downstream products of the PLA₂ pathway such as arachidonic acid metabolites, including an array of eicosanoids as well as reactive oxygen species, mediate deleterious effects with a potential role in the ensuing lethal response.

The results of a study currently in progress demonstrate that activation of the PLA₂ pathway mediated by endogenous peroxynitrite is a critical event leading to mitochondrial dysfunction that is causally linked to necrotic PC12 cell death. Indeed, we found that the peroxynitrite-dependent lethal response was blunted by low concentrations of two structurally unrelated PLA₂ inhibitors. These effects were downstream to NO and peroxynitrite formation because each of these inhibitors failed to inhibit NO formation and nitration of tyrosine. In addition, nanomolar levels of arachidonic acid restored the lethal response in NOS- or PLA₂-inhibited cells. Finally, the decline in cellular ATP mediated by endogenous peroxynitrite was prevented by PLA₂ inhibitors, and the concomitant addition of arachidonic acid reversed this effect. Thus, these results lead to the identification of a cytoprotective strategy to counteract the deleterious effects mediated by peroxynitrite. This conclusion has a number of important implications because it may provide the basis for a novel therapeutic approach for an array of pathologies in which peroxynitrite cytotoxicity plays a critical role. The conventional strategies to counteract the deleterious effects mediated by peroxynitrite, based on scavenging or preventing its formation (20), could be supplemented by the use of pharmacologic inhibitors of the signaling pathway involved in the peroxynitrite-dependent lethal response.

It is important, however, to emphasize that our findings were obtained using a specific toxicity paradigm, and further studies are necessary to determine the generality of the observed effects. It is likely that highly reactive molecules such as peroxynitrite and other reactive oxygen species have the ability to promote cell death by multiple and eventually synergistic mechanisms. For obvious reasons, the deleterious effects mediated by these species will be largely influenced by both their concentration and the site of formation. This implies that different mechanisms may lead to toxicity after exposure to a given toxic agent in various cell types expressing constitutive NOS activity in different amounts and locations.

Thus, although our results identify an important toxicological role for the PLA₂ pathway stimulated by endogenous peroxynitrite, future studies should investigate whether the same mechanism operates in additional biological settings, including cells in primary culture as well as experimental animals.

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