Cell Signaling and Cytotoxicity by Peroxynitrite

Orazio Cantoni, Letizia Palomba, Andrea Guidarelli, Ilaria Tommasini, Liana Cerioni, and Piero Sestili

Istituto di Farmacologia e Farmacognosia, Università degli Studi di Urbino, Urbino, Italy

Reactive nitrogen species are now considered to play an important role in various pathologies. Although the pathological significance of these molecules, peroxynitrite in particular, has long been attributed to their abilities to react with any component of the cells, including lipids, proteins, and DNA, a paradigm shift has recently been occurring whereby reactive nitrogen species are appreciated as signaling molecules. The question therefore arises as to whether nitrosative stress is indeed the result of a random (stochastic) process of cell damage, as it has traditionally been viewed, or rather is a consequence of the specific activation of a cascade of signaling events. The above considerations have provided the bases for the research work performed in our laboratory, and the results obtained are illustrated in the present article. In particular, our results indicate that some effects of peroxynitrite are not directly mediated by the oxidant; rather, it appears that peroxynitrite triggers a signaling pathway that finally leads to cytotoxicity. *Key words:* **cell death, mitochondrial permeability transition, nitric oxide, peroxynitrite, phospholipase A_2.** *Environ Health Perspect* **110(suppl 5):823–825 (2002).**
http://ehpnet1.niehs.nih.gov/docs/2002/suppl-5/823-825cantoni/abstract.html

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 cell 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 endotoxemia, neurological disorders, atherosclerosis, and ischemia/reperfusion (*3,5*). Although NO can be directly detrimental to target cells, most of its toxic effects appear to be mediated by peroxynitrite, the coupling product of NO and superoxides (*5–7*). The cytotoxic potential of peroxynitrite has long been attributed to its ability to react with all the major classes of biomolecules (*8–11*). Indeed, peroxynitrite causes an array of effects, including lipid peroxidation (*12*), protein nitration and nitrosylation (*13*), DNA damage (*9,10,14*), and oxidation of thiols (*15*), which most likely represent upstream events leading to inhibition of mitochondrial respiration (*5,14,16,17*), mitochondrial permeability transition (*18*), and/or other dysfunctions promoting cell death.

Thus, unraveling the exact role of each of the lesions generated by peroxynitrite in the context of cell death is not an easy task, and as a consequence, the ensuing lethal response has traditionally been viewed as the result of a stochastic process of cell damage.

An obvious consequence of the above premise is that the strategies to mitigate the deleterious effects mediated by peroxynitrite are restricted to the use of scavengers of this species (*19,20*) and to agents inhibiting its formation (e.g., superoxide dismutase mimetics or NOS inhibitors) (*20–22*), which all present some important limitations when used *in vivo*.

It is important to note, however, that a paradigm shift has recently been occurring whereby reactive nitrogen species are appreciated as signaling molecules (*23,24*). The identification of events triggered by peroxynitrite and leading to cytotoxicity would therefore allow the development of cytoprotective strategies targeted downstream to peroxynitrite.

Cell Signaling Induced by Peroxynitrite

Accumulating evidence suggests that various reactive oxygen and nitrogen species, including peroxynitrite, serve several physiological or pathological functions. In particular, peroxynitrite was recently shown to upregulate src tyrosine kinases (*25*) as well as the phosphoinositide 3-kinase Akt pathway (*26*). A large number of studies investigated the effects of peroxynitrite on mitogen-activated protein kinases (*27–30*), a family of serine/threonine kinases that regulate an array of cellular activities. It was found that the three major subfamilies, extracellular signal-regulated kinases, p38 mitogen–activated protein kinases, and c-Jun NH_2-terminal kinases, are activated by peroxynitrite. Because mitogen-activated protein kinases, p38 mitogen–activated protein kinase and c-Jun NH_2-terminal kinase in particular, are implicated in apoptosis, the possibility exists that these responses play a major role in the process of peroxynitrite-induced cell death.

We recently reported that both endogenous and exogenous peroxynitrite effectively promotes a release of arachidonic acid mediated by stimulation of phospholipase A_2 (PLA_2) activity in PC12 cells (*31*). This 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 PLA_2. These results therefore suggest that downstream products of the PLA_2 pathway may play a role in the lethal response evoked by peroxynitrite.

Cell Death Induced by Peroxynitrite

Apoptosis is the most frequently reported mode of peroxynitrite-induced cell death (*32–40*); other studies, however, have shown that peroxynitrite leads to necrosis (*41*) or to both modes of cell death (*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 (*5,16*), 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

This article is part of the monograph *Molecular Mechanisms of Metal Toxicity and Carcinogenicity.*

Address correspondence to O. Cantoni, Istituto di Farmacologia e Farmacognosia, Università di Urbino, Via S. Chiara, 27-61029, Urbino (PU), Italy. Telephone: 39-0722-2671. Fax: 39-0722-327670. E-mail: cantoni@uniurb.it

The financial support of Progetti di Ricerca di Interesse Nazionale (O.C.) is gratefully acknowledged.

Received 26 February 2002; accepted 20 May 2002.

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 clear-cut 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 PLA_2. 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 PLA_2 inhibitors; *b*) low levels of authentic peroxynitrite restored the DHR fluorescence response in NOS-inhibited cells but not in PLA_2-inhibited cells, whereas reagent arachidonic acid was effective under both conditions; *c*) the DHR fluorescence response induced by authentic peroxynitrite was also blunted by PLA_2 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 PLA_2 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 PLA_2 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 PLA_2 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 PLA_2-inhibited cells. Finally, the decline in cellular ATP mediated by endogenous peroxynitrite was prevented by PLA_2 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 PLA_2 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.

References and Notes

1. Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem 63:175–195 (1994).
2. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J 298:249–258 (1994).
3. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 43:109–142 (1991).
4. Stamler JS. Redox signaling: nitrosylation and related target interactions of nitric oxide. Cell 78:931–936 (1994).
5. Heales SJ, Bolanõs JP, Stewart VC, Brookes PS, Land JM, Clark JB. Nitric oxide, mitochondria and neurological disease. Biochim Biophys Acta 1410:215–228 (1999).
6. Beckman JS. The double-edged role of nitric oxide in brain function and superoxide-mediated injury. J Dev Physiol 15:53–59 (1991).
7. Murphy MP. Nitric oxide and cell death. Biochim Biophys Acta 1411:401–414 (1999).
8. Rubbo H, Radi R, Trujillom M, Kalyanaraman B, Barnes S, Kirk M, Freeman BA. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. J Biol Chem 269:26066–26075 (1994).
9. Salgo MG, Bermudez E, Squadrito GL, Pryor WA. Peroxynitrite causes DNA damage and oxidation of thiols in rat thymocytes. Arch Biochem Biophys 322:500–505 (1995).
10. Szabó C, Ohshima H. DNA damage induced by peroxynitrite: subsequent biological effects. Nitric Oxide 1:373–385 (1997).
11. Eiserich JP, Patel RP, O'Donnell VB. Pathophysiology of nitric oxide and related species: free radical reactions and modification of biomolecules. Mol Aspects Med 19:221–357 (1998).
12. Radi R, Beckman JS, Bush HM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys 288:481–487 (1991).
13. Patel RP, McAndrew J, Sellak H, White CR, Jo H, Freeman BA, Darley-Usmar VM. Biological aspects of reactive nitrogen species. Biochim Biophys Acta 1411:385–400 (1999).
14. Guidarelli A, Tommasini I, Fiorani M, Cantoni O. Essential role of the mitochondrial respiratory chain in peroxynitrite-induced strand scission of genomic DNA. IUBMB Life 50:195–201(2000).

15. Quijano C, Alvarez B, Gatti RM, Augusto O, Radi R. Pathways of peroxynitrite oxidation of thiol groups. Biochem J 322:167–173 (1997).
16. Bolanõs JP, Heales SJ, Land JM, Clark JB. Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurones and astrocytes in primary culture. J Neurochem 64:1965–1972 (1995).
17. Cassina A, Radi R. Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. Arch Biochem Biophys 328:309–316 (1996).
18. Packer MA, Scarlett JL, Martin SW, Murphy MP. Induction of the mitochondrial permeability transition by peroxynitrite. Biochem Soc Trans 25:909–914 (1997).
19. Salgo MG, Pryor WA. Trolox inhibits peroxynitrite-mediated oxidative stress and apoptosis in rat thymocytes. Arch Biochem Biophys 333:482–488 (1996).
20. Arteel GE, Briviba K, Sies H. Protection against peroxynitrite. FEBS Lett 445:226–230 (1999).
21. Pfeiffer S, Schrammel A, Koesling D, Schmidt K, Mayer B. Molecular actions of a Mn(III)porphyrin superoxide dismutase mimetic and peroxynitrite scavenger: reaction with nitric oxide and direct inhibition of NO synthase and soluble guanylyl cyclase. Mol Pharmacol 53:795–800 (1998).
22. Salvemini D, Riley DP, Lennon PJ, Wang ZQ, Currie MG, Macarthur H, Misko TP. Protective effects of a superoxide dismutase mimetic and peroxynitrite decomposition catalysts in endotoxin-induced intestinal damage. Br J Pharmacol 127:685–692 (1999).
23. Khan AU, Wilson T. Reactive oxygen species as cellular messengers. Chem Biol 2:437–445 (1995).
24. Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free Radic Biol Med 28:1456–1462 (2000).
25. Malozzi C, Di Stasi AM, Minetti M. Activation of src tyrosine kinases by peroxynitrite. FEBS Lett 456:201–206 (1999).
26. Klotz LO, Schieke SM, Sies H, Holbrook NJ. Peroxynitrite activates the phosphoinositide 3-kinase/Akt pathway in human skin primary fibroblast. Biochem J 252:219–225 (2000).
27. Schieke SM, Briviba K, Klotz L-O, Sies H. Activation pattern of mitogen-activated protein kinases elicited by peroxynitrite: attenuation by selenite supplementation. FEBS Lett 448:301–303 (1999).
28. Jope RS, Zhang L, Song L. Peroxynitrite modulates the activation of p38 and extracellular regulated kinases in PC12 cells. Arch Biochem Biophys 376:365–370 (2000).
29. Zhang P, Wang Y-Z, Kagan E, Bonner JC. Peroxynitrite targets the epidermal growth factor receptor, Raf-1 and MEK independently to activate MAPK. J Biol Chem 275:22479–22486 (2000).
30. Bapat S, Verkleij A, Post JA. Peroxynitrite activates mitogen-activated protein kinase (MAPK) via a MEK-independent pathway: a role for protein kinase C. FEBS Lett 499:21–26 (2001).
31. Guidarelli A, Palomba L, Cantoni O. Peroxynitrite-mediated release of arachidonic acid from PC12 cells. Br J Pharmacol 129:1539–1542 (2000).
32. Lin KT, Xue JY, Nomen M, Spur B, Wong PY. Peroxynitrite-induced apoptosis in HL-60 cells. J Biol Chem 270:16487–16490 (1995).
33. Shin JT, Barbeito L, MacMillan-Crow LA, Beckman JS, Thompson JA. Acidic fibroblast growth factor enhances peroxynitrite-induced apoptosis in primary murine fibroblasts. Arch Biochem Biophys 335:32–41 (1996).
34. Szabó C. DNA strand breakage and activation of poly-ADP ribosyltransferase: a cytotoxic pathway triggered by peroxynitrite. Free Radic Biol Med 21:855–869 (1996).
35. Lin KT, Xue JY, Sun FF, Wong PY. Reactive oxygen species participate in peroxynitrite-induced apoptosis in HL-60 cells. Biochem Biophys Res Commun 230:115–119 (1997).
36. Virág L, Marmer DJ, Szabó C. Crucial role of apopain in the peroxynitrite-induced apoptotic DNA fragmentation. Free Radic Biol Med 25:1075–1082 (1998).
37. Virág L, Scott GS, Cuzzocrea S, Marmer D, Salzman AL, Szabó C. Peroxynitrite-induced thymocyte apoptosis: the role of caspases and poly(ADP-ribose) synthetase (PARS) activation. Immunology 94:345–355 (1998).
38. Foresti R, Sarathchandra P, Clark JE, Green CJ, Motterlini R. Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis. Biochem J 339:729–736 (1999).
39. Oh-hashi K, Maruyama W, Yi H, Takahashi T, Naoi M, Isobe K. Mitogen-activated protein kinase pathway mediates peroxynitrite-induced apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells. Biochem Biophys Res Commun 263:504–509 (1999).
40. Virág L, Scott GS, Antal-Szalmás P, O'Connor M, Ohshima H, Szabó C. Requirement of intracellular calcium mobilization for peroxynitrite-induced poly(ADP-ribose)synthetase activation and cytotoxicity. Mol Pharmacol 56:824–833 (1999).
41. Delaney CA, Tyrberg B, Bouwens L, Vaghef H, Hellman B, Eizirik DL. Sensitivity of human pancreatic islets to peroxynitrite-induced cell dysfunction and death. FEBS Lett 394:300–306 (1996).
42. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-*D*-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci USA 92:7162–7166 (1995).
43. Estévez AG, Radi R, Barbeito L, Shin JT, Thompson JA, Beckman JS. Peroxynitrite-induced cytotoxicity in PC12 cells: evidence for an apoptotic mechanism differentially modulated by neurotrophic factors. J Neurochem 65:1543–1550 (1995).
44. Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem 266:4244–4250 (1991).
45. Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am J Physiol 268:699–722 (1995).
46. Sestili P, Tommasini I, Cantoni O. Peroxynitrite promotes mitochondrial permeability transition-dependent rapid U937 cell necrosis: survivors proliferate with kinetics superimposable on those of untreated cells. Free Radic Res 34:513–527 (2001).
47. Sestili P, Clementi E, Guidarelli A, Sciorati C, Cantoni O. Endogenous and exogenous nitric oxide enhance the DNA strand scission induced by *tert*-butylhydroperoxide in PC12 cells via peroxynitrite-dependent and independent mechanisms, respectively. Eur J Neurosci 12:145–154 (2000).
48. Palomba L, Sestili P, Cantoni O. *tert*-Butylhydroperoxide induces peroxynitrite-dependent mitochondrial permeability transition leading PC12 cells to necrosis. J Neurosci Res 65:387–395 (2001).
49. Palomba L, Sestili P, Guidarelli A, Sciorati C, Clementi E, Fiorani M, Cantoni O. Products of the phospholipase A_2 pathway mediate the dihydrorhodamine fluorescence response evoked by endogenous and exogenous peroxynitrite in PC12 cells. Free Radic Biol Med 29:783–789 (2000).