Peroxynitrite modulates tyrosine phosphorylation and phosphoinositide signalling in human neuroblastoma SH-SY5Y cells: attenuated effects in human 1321N1 astrocytoma cells

Xiaohua LI, Patrizia DE SARNO, Ling SONG, Joseph S. BECKMAN and Richard S. JOPE¹

Department of Psychiatry and Behavioral Neurobiology, Sparks Center 1057, University of Alabama at Birmingham, Birmingham, AL 35294-0017, U.S.A.

Peroxynitrite may contribute to oxidative stress involving neurodegeneration in several disorders, including Alzheimer's disease. As with other reactive oxygen species, peroxynitrite might affect neuronal signalling systems, actions that could contribute to adaptive or deleterious cellular outcomes, but such effects have not previously been studied. To address this issue directly, peroxynitrite (50–500 μ M) was administered to human neuroblastoma SH-SY5Y cells to assess its effects on protein tyrosine nitration, phosphoinositide signalling and protein tyrosine phosphorylation. Peroxynitrite rapidly increased the nitro-tyrosine immunoreactivity of numerous proteins, primarily in the cytosol. Peroxynitrite inhibited, in a concentration-dependent manner, phosphoinositide hydrolysis stimulated by activation of muscarinic receptors with carbachol and the inhibition was greater after the depletion of cellular glutathione. In comparison,

muscarinic receptor-stimulated phosphoinositide hydrolysis in human astrocytoma 1321N1 cells was less vulnerable to inhibition by peroxynitrite either without or with prior depletion of glutathione. There was a large, rapid and reversible increase in the tyrosine phosphorylation of the p120 Src substrate in peroxynitrite-treated SH-SY5Y cells, a response that was potentiated by glutathione depletion; in contrast, peroxynitrite decreased the tyrosine phosphorylation of focal adhesion kinase and paxillin. Tyrosine phosphorylation of p120 in 1321N1 astrocytoma cells was less sensitive to modulation by peroxynitrite. Thus alterations in phosphoinositide signalling and protein tyrosine phosphorylation were greater in neuroblastoma than astrocytoma cells, and modulation of these signalling processes probably contributes to neuronal mechanisms of the response to peroxynitrite.

INTRODUCTION

Oxidative stress is widely recognized as an important pathophysiological mechanism contributing to many neurodegenerative conditions [1–4]. One of the important agents that mediates oxidative stress-induced impairments of neuronal function seems to be peroxynitrite, a potent oxidant that is the product of the near-diffusion-limited reaction of superoxide with nitric oxide [5]. Peroxynitrite has been implicated in the neuropathology of several neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, and ischaemia/reperfusion injury [6,7], in part from evidence, in the post-mortem brain of protein tyrosine nitration, a signature product of peroxynitrite-mediated modification of protein tyrosine residues [8–13]. Thus peroxynitrite might be a critical agent contributing to impaired neuronal function caused by excessive oxidative stress.

Reactive oxygen species such as H_2O_2 and hydroxyl radicals influence cellular signalling processes such as protein tyrosine phosphorylation and second-messenger systems both as integral components of signalling pathways and as neurotoxic mediators of oxidative stress [14]. For example, H_2O_2 alters protein tyrosine phosphorylation and inhibits receptor-coupled phosphoinositide signal transduction activity [15,16]. Thus these signalling processes affected by oxidative agents represent potential targets for the actions of peroxynitrite. Indeed, two recent reports demonstrated that protein tyrosine phosphorylation in intact endothelial cells and human platelets was modulated by peroxynitrite [17,18]. However, little is known about the effects of peroxynitrite on signalling systems in neuronal cells. In PC12 cells, inhibition of

nerve growth factor-induced activation of phosphatidylinositol 3-kinase was evident 24 h after exposure to 650 μ M peroxynitrite [19]. Although little else has been reported concerning the direct effects of peroxynitrite on neuronal signalling processes, it is quite evident that peroxynitrite can have severe deleterious effects. For example, apoptosis was observed in 35% of PC12 cells 24 h after exposure to 650 μ M peroxynitrite [19,20]. Peroxynitrite also impaired neuronal mitochondrial function [21]. Furthermore astrocytes were comparatively resistant to impaired mitochondrial function caused by peroxynitrite, as no inhibition in cultured astrocytes was observed with 1 mM peroxynitrite, which significantly impaired mitochondrial function in cultured neurons, unless cellular glutathione was depleted beforehand [22,23]. Although seemingly high concentrations of peroxynitrite have been used in most of these studies *in vitro*, the decomposition of peroxynitrite is rapid at pH 7.4, such that little actually comes in contact with culture cells even with an initial concentration of 1 mM peroxynitrite [19]. Thus these studies *in vitro* are widely believed to model the effects of physiologically relevant concentrations of peroxynitrite *in vivo*.

The association of peroxynitrite with several prevalent neurodegenerative conditions and the known susceptibility of the phosphoinositide signalling system and of protein tyrosine phosphorylation signalling to oxidative agents led us to test whether these signalling processes were affected by peroxynitrite. Human neuroblastoma SH-SY5Y cells were used in this study because this is a widely used cell line for modelling neuronal characteristics and because these cells express M3 muscarinic receptors coupled to the phosphoinositide signal transduction system [24]. In addition, human astrocytoma 1321N1 cells were used for com-

Abbreviations used: BSO, buthionine sulfoximine; PAO, phenylarsine oxide.

¹ To whom correspondence should be addressed.

parative studies because they also express M3 muscarinic receptors coupled to phosphoinositide signalling [25]. The results demonstrate that neuronal phosphoinositide signalling is susceptible to inhibition by relatively low concentrations of peroxynitrite, that this impairment is greater after glutathione depletion, that astrocyte phosphoinositide signalling is less impaired by peroxynitrite even after glutathione depletion, and that the neuronal tyrosine phosphorylation of proteins can be increased or decreased by peroxynitrite in a protein-specific and time-dependent manner.

EXPERIMENTAL

Materials

Commercial reagents were obtained as follows: RPMI medium from Cellgro (Herndon, VA, U.S.A.); Dulbecco's modified Eagle's medium and fetal bovine serum from Gibco-BRL (Gaithersburg, MD, U.S.A.); fetal clone II from Hyclone (Logan, UT, U.S.A.); focal adhesion kinase, paxillin and phosphotyrosine antibodies from Transduction Laboratories (Lexington, KY, U.S.A.); p120 antibody from Upstate Biotechnology (Lake Placid, NY, U.S.A.); Protein A-Sepharose 6MB from Pharmacia (Piscataway, NJ, U.S.A.), buthionine sulfoximine (BSO) from Sigma (St. Louis, MO, U.S.A.); and *myo*-[³H]inositol from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). The antibody against nitrotyrosine was described previously [26]. Stock 100 mM peroxynitrite was synthesized as described [26] and was added directly to the medium in culture dishes or to assay buffer containing cells (followed by rapid mixing) to avoid degradation of peroxynitrite. The SH-SY5Y and 1321N1 cells were generously provided by Dr. Stephen Fisher (University of Michigan, Ann Arbor, MI, U.S.A.) and Dr. Joan H. Brown (University of California, San Diego, CA, U.S.A.) respectively.

Cell culture

Human neuroblastoma SH-SY5Y cells were grown in RPMI medium containing 10% (v/v) horse serum, 5% (v/v) fetal clone II, 2 mM L-glutamine, 100 i.u./ml penicillin and 100 µg/ml streptomycin. Human astrocytoma 1321N1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 i.u./ml penicillin and 100 µg/ml streptomycin. Cells were maintained in humidified, 37 °C chambers with air/CO₂ (19:1). Cells were plated at a density of approx 2 × 10⁵ cells per 100 mm dish and were harvested approx. 48 h later, after the treatments described in the Results section. Glutathione concentrations were measured by the method of Tietze [27].

Phosphoinositide hydrolysis

Cells were prelabelled with 7.5 µCi/ml *myo*-[2-³H]inositol for 48 h, harvested, resuspended in buffer [30 mM Hepes (pH 7.4)/122 mM NaCl/3.6 mM NaHCO₃/1.2 mM KH₂PO₄/1.2 mM MgCl₂/5 mM KCl/1.3 mM CaCl₂/10 mM LiCl/11 mM glucose] and washed twice, as described previously [16]. Suspended cells were preincubated for 5 min with or without peroxynitrite followed by incubation with 1 mM carbachol for 15 min, or with 20 mM NaF (with 10 µM AlCl₃) or 50 µM ionomycin (with 2.2 mM CaCl₂) for 30 min at 37 °C. Radioactivity was measured in samples after the fractionation of lipids, inositol monophosphate and inositol as described previously [16]. Measurements were made in triplicate for each treatment in at least three separate experiments. Statistical significance was determined by using analysis of variance (ANOVA) with a post-hoc Dunnett

multiple comparison test, except for comparisons of matching treatments with or without BSO pretreatment, when Student's *t* test was used.

Immunoprecipitation and immunoblotting

Cells were washed twice with PBS and were lysed with buffer A [20 mM Tris (pH 8.0)/137 mM NaCl/2 mM EDTA/10% (v/v) glycerol/1 mM PMSF/10 µg/ml aprotinin/10 µg/ml leupeptin/1 mM sodium vanadate/1% (v/v) Triton X-100]. After a 30 min incubation on ice, the lysates were centrifuged at 10000 *g* for 10 min to remove detergent-insoluble material. Protein concentrations were determined by the method of Bradford [28].

To separate cytosolic and membrane proteins, washed cells were harvested in TE buffer [20 mM Tris (pH 8.0)/2 mM EDTA/1 mM PMSF/10 µg/ml aprotinin/10 µg/ml leupeptin/1 mM sodium vanadate]. After 30 min on ice, samples were pulse-sonicated three times (0.3 relative output) for 5 s, then centrifuged at 40000 *g* for 30 min at 4 °C. The supernatant was used as the cytosolic fraction. The pellets were washed twice with TE buffer, proteins were extracted with buffer A as described for whole cell lysates, and the resulting supernatant was used as the membrane fraction.

For immunoprecipitation, cell lysates (0.5 mg of protein/ml of buffer A) were incubated overnight with primary antibody and 3 mg of washed Protein A-Sepharose. For nitrotyrosine and phosphotyrosine measurements, cell lysates (25 µg of protein) were used directly. Samples were boiled in Laemmli [29] sample buffer containing 2% (w/v) SDS for 5 min, proteins were resolved by SDS/PAGE [7.5% (w/v) gel] and transferred to nitrocellulose; immunoblots were developed by enhanced chemiluminescence. All treatments were tested at least three times with different batches of cells.

RESULTS

Protein nitration

Tyrosine nitration, a signature of peroxynitrite-modified protein tyrosine residues, was measured in lysates of SH-SY5Y cells by immunoblotting with an antibody that specifically recognizes nitrotyrosine. Although stable in stock basic solutions, peroxynitrite has a short half-life at physiological pH, so initial responses to peroxynitrite would be expected to occur rapidly. Exposure of cells to 200 µM peroxynitrite rapidly increased the nitrotyrosine immunoreactivity from a few bands that were detected in untreated cells to numerous bands within 5 min of peroxynitrite treatment (Figure 1A). This protein modification seemed to reach completion rapidly and to be stable, because nitrotyrosine immunoreactivity was essentially unchanged in cell lysates prepared after longer times of incubation after peroxynitrite addition. The extent of protein tyrosine nitration was dependent on the concentration of peroxynitrite (Figure 1B). Increased nitrotyrosine immunoreactivity above control levels was evident in cells exposed to 50 µM peroxynitrite, the nitrotyrosine content was greater after the addition of 200 µM peroxynitrite and there was an exceptionally large increase in nitrotyrosine immunoreactivity after treatment with 500 µM peroxynitrite. These results demonstrate that the action of peroxynitrite is rapid, so incubation times in the 5–10 min range were used in further experiments, and that 200 µM peroxynitrite is an appropriate concentration to use to achieve moderate effects.

Cytosolic and membrane fractions were prepared and examined for nitrotyrosine immunoreactivity to determine whether peroxynitrite penetrated cells. After a 10 min incubation of cells with 200 µM peroxynitrite, the increased nitrotyrosine immuno-

Figure 1 Increase in protein nitrotyrosine immunoreactivity induced in SH-SY5Y cells by peroxynitrite

(A) Cells were incubated for 5, 10, 15, 20 or 30 min after the addition of 200 μ M peroxynitrite. (B) Cells were incubated with 0, 50, 100, 200 or 500 μ M peroxynitrite (PN) for 15 min. Cells were lysed, proteins (25 μ g) were resolved by electrophoresis and nitrotyrosine immunoreactivity was determined as described in the Experimental section. Panel (B) is a lighter exposure than (A) to permit the distinction of individual bands after treatment with 500 μ M peroxynitrite. Results shown are representative of three individual experiments. The positions of molecular mass standards are shown (in kDa) at the right.

reactivity was found to be almost completely associated with cytosolic proteins (Figure 2). This result confirmed that peroxynitrite permeated SH-SY5Y cells and could modify intracellular proteins.

Phosphoinositide hydrolysis

The next goal was to test whether peroxynitrite influenced the activity of the phosphoinositide signal transduction system. SH-SY5Y cells that had been prelabelled with [3 H]inositol were

Figure 2 Effects of peroxynitrite on protein nitrotyrosine in cytosol and membrane fractions from SH-SY5Y cells

Cells were untreated or incubated for 10 min after the addition of 200 μ M peroxynitrite (PN), followed by the separation of cytosol and membrane fractions and the measurements of nitrotyrosine immunoreactivity after protein (25 μ g) electrophoresis as described in the Experimental section. Data are representative results from three individual experiments. The positions of molecular mass standards are shown (in kDa) at the right.

exposed to peroxynitrite; after 5 min, 1 mM carbachol was added to stimulate M3 muscarinic receptors that are coupled to activation of the phosphoinositide signal transduction system in these cells. Carbachol induced a 4-fold increase in phosphoinositide hydrolysis compared with the basal, unstimulated rate, and this response was inhibited by peroxynitrite in a concentration-dependent manner (Figure 3A). Peroxynitrite at concentrations of 50, 100, 200 and 300 μ M inhibited the response to carbachol by 11%, 28%, 66% and 73% respectively with statistically significant ($P < 0.05$) inhibitions achieved with the three highest concentrations of peroxynitrite. In the absence of carbachol, the basal rate of phosphoinositide hydrolysis was only slightly decreased by peroxynitrite [e.g. a $15 \pm 7\%$ (S.E.M.) decrease was obtained with 300 μ M peroxynitrite].

To test whether endogenous glutathione modulated the inhibitory effects of peroxynitrite on phosphoinositide hydrolysis, glutathione was depleted by incubating cells with 200 μ M BSO for 48 h, during the prelabelling incubation with [3 H]inositol. This treatment with BSO did not affect the uptake or incorporation of [3 H]inositol into lipids (Table 1), and it decreased the glutathione concentration from 3.66 ± 0.08 ng/ μ g of protein ($n = 3$) in control cells to undetectable levels. By itself, BSO treatment had no significant effect on carbachol-stimulated phosphoinositide hydrolysis (Table 1), but it significantly potentiated the inhibitory effect of peroxynitrite on carbachol-induced phosphoinositide hydrolysis (Figure 3A).

Human astrocytoma 1321N1 cells, which express M3 muscarinic receptors that are coupled to the phosphoinositide signalling system, were used to determine whether there was a difference in the inhibitory effect of peroxynitrite on carbachol-induced phosphoinositide hydrolysis compared with that occurring in human neuroblastoma SH-SY5Y cells. 1321N1 cells that had been prelabelled with [3 H]inositol were exposed to peroxynitrite (50–300 μ M) for 5 min before the addition of 1 mM carbachol.

Figure 3 Peroxynitrite inhibition of carbachol-induced phosphoinositide (PI) hydrolysis

Human neuroblastoma SH-SY5Y cells (A) or human astrocytoma 1321N1 cells (B) were prelabelled with [3 H]inositol for 48 h in the absence (controls, ●) or presence of 200 μ M BSO (■). Prelabelled cells were suspended in assay buffer, incubated for 5 min with 0, 50, 100, 200 or 300 μ M peroxynitrite followed by 15 min with 1 mM carbachol; the production of [3 H]inositol monophosphate was measured. Results are shown as the percentage of values from controls treated with carbachol in the absence of peroxynitrite, and are means $\pm$ S.E.M. for three experiments, with each treatment measured in triplicate. * Significantly different from carbachol stimulation in cells not exposed to peroxynitrite or BSO (100%), $P < 0.05$ by ANOVA; † significantly different from matched cells treated with peroxynitrite but not with BSO, $P < 0.05$ by Student's t test.

Phosphoinositide hydrolysis was stimulated 4-fold over basal by carbachol, similar to the stimulation observed in SH-SY5Y cells (Table 1). Peroxynitrite pretreatment inhibited this response to carbachol, but to a smaller extent than the inhibition that it caused in SH-SY5Y cells (Figure 3B). For example, the inhibition by 200 μ M peroxynitrite was only 28 % in 1321N1 cells, whereas this concentration caused a 66 % inhibition in SH-SY5Y cells. The basal rate of phosphoinositide hydrolysis was unaffected by peroxynitrite (e.g. it was $99 \pm 4\%$ of control with 300 μ M peroxynitrite). Pretreatment of 1321N1 cells with 200 μ M BSO decreased glutathione levels from 8.34 ± 0.70 ng/ μ g of protein to 0.09 ± 0.03 ng/ μ g of protein ($N = 3$), and this did not significantly alter the incorporation of [3 H]inositol into lipids or the basal or carbachol-stimulated hydrolysis of phosphoinositides (Table 1). However, pretreatment with BSO only slightly potentiated the inhibitory effect of peroxynitrite on carbachol-induced phospho-

Table 1 [3 H]inositol distribution unaffected by BSO

Cells were prelabelled with [3 H]inositol for 48 h in the absence (control) or presence of 200 μ M BSO. Prelabelled cells were suspended in assay buffer, preincubated for 5 min then incubated for 15 min without (basal) or with 1 mM carbachol; [3 H]inositol was measured in the indicated fractions as described in the Experimental section. Values are means $\pm$ S.E.M. There were no statistically significant differences between control and BSO-treated cells. Abbreviation: IP₁, inositol monophosphate.

Cells	Fraction	Inositol (c.p.m.)	
		Control	BSO
SH-SY5Y	Inositol	83 827 $\pm$ 12 652	101 799 $\pm$ 26 443
	Lipids	23 277 $\pm$ 3174	21 012 $\pm$ 4514
	IP ₁ (basal)	863 $\pm$ 146	983 $\pm$ 223
	IP ₁ (carbachol)	3743 $\pm$ 443	3234 $\pm$ 736
1321N1	Inositol	448 912 $\pm$ 41 484	449 832 $\pm$ 63 364
	Lipids	105 687 $\pm$ 8965	98 882 $\pm$ 4611
	IP ₁ (basal)	1981 $\pm$ 119	2112 $\pm$ 490
	IP ₁ (carbachol)	8571 $\pm$ 158	8266 $\pm$ 206

inositide hydrolysis in 1321N1 cells. Thus M3 muscarinic receptor-stimulated phosphoinositide hydrolysis was less susceptible to inhibition by peroxynitrite in human astrocytoma 1321N1 cells than in human neuroblastoma SH-SY5Y cells, and the resistance of astrocytoma cells was not due only to their higher glutathione levels.

To examine whether peroxynitrite affects the activation of the G-proteins ($G_{q/11}$) or phospholipase C, which mediate carbachol-stimulated phosphoinositide signalling, SH-SY5Y cells were incubated with 20 mM NaF to activate $G_{q/11}$ directly, or with 50 μ M ionomycin, a Ca^{2+} ionophore, to increase intracellular Ca^{2+} concentrations to activate phospholipase C directly. Peroxynitrite only slightly inhibited phosphoinositide hydrolysis induced by direct activation of G-proteins by NaF (Figure 4A), and peroxynitrite had no significant effect on phosphoinositide hydrolysis induced by the stimulation of phospholipase C by ionomycin treatment (Figure 4B). These results indicate that phospholipase C and the lipid substrates were not directly affected by peroxynitrite but that receptor activation leading to stimulation of $G_{q/11}$ and phospholipase C was impaired.

Protein tyrosine phosphorylation

We next tested whether peroxynitrite treatment of SH-SY5Y cells modulated protein tyrosine phosphorylation. Treatment with 200 μ M peroxynitrite caused a rapid increase in the phosphotyrosine immunoreactivity of several protein bands, such as those migrating at 190, 120, 95, 85, 65, 58 and 50 kDa (Figure 5A). The maximal increases in protein tyrosine phosphorylation occurred within 5–10 min of exposure of the cells to peroxynitrite. The initial increase in phosphotyrosine immunoreactivity was followed temporally by a decrease, so that within 20–30 min of peroxynitrite addition there was a generalized decrease in protein tyrosine phosphorylation to levels lower than those observed in untreated cells. This rapid increase after exposure to peroxynitrite in protein tyrosine phosphorylation followed by a decline is similar to the biphasic response in phosphotyrosine immunoreactivity recently reported in human platelets exposed to peroxynitrite [18]. The stimulation of phosphotyrosine immunoreactivity induced by peroxynitrite was even more evident with a higher concentration (500 μ M) of peroxynitrite, 15 min after treatment, although by this time the stimulation caused by lower concentrations of peroxynitrite was diminished (Figure 5B).

Figure 4 Effects of peroxynitrite on phosphoinositide (PI) hydrolysis stimulated by NaF or ionomycin in SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells were prelabelled with [3 H]inositol for 48 h. Prelabelled cells were suspended in assay buffer, incubated for 5 min with 0, 50, 100, 200 or 300 μ M peroxynitrite followed by 30 min with 20 mM NaF plus 10 μ M AlCl_3 (A) or 50 μ M ionomycin with 2.2 mM CaCl_2 in the buffer (B), and the production of [3 H]inositol monophosphate was measured. Results are shown as the percentage of values from controls treated with NaF or ionomycin in the absence of peroxynitrite, and are means $\pm$ S.E.M. for three experiments, with each treatment measured in triplicate. *Significantly different from cells not exposed to peroxynitrite (100%), $P < 0.05$ by ANOVA.

With 500 μ M peroxynitrite, both the magnitude and the number of phosphotyrosine immunoreactive bands were increased compared with the effects of lower concentrations of peroxynitrite. Thus peroxynitrite can cause increases in protein tyrosine phosphorylation that occur rapidly and are greater with higher concentrations of peroxynitrite, but there is a subsequent decrease in phosphotyrosine immunoreactivity and the temporal pattern varies among the affected proteins.

To obtain an indication of whether peroxynitrite increased tyrosine phosphorylation by activating kinases or inhibiting phosphatases, the tyrosine phosphatase inhibitor phenylarsine oxide (PAO) was used. Preliminary experiments of the PAO concentration dependence (3–30 μ M) indicated that a concentration of 20 μ M PAO caused a large increase in phosphotyrosine immunoreactivity. Figure 6 shows that a 5 min pretreatment with 200 μ M peroxynitrite potentiated PAO-induced increases in phosphotyrosine immunoreactivity, most notably of the 180, 95

Figure 5 Modulation of tyrosine phosphorylation by peroxynitrite in SH-SY5Y cells

(A) Peroxynitrite (200 μ M) was added and cells were incubated for 5, 10, 15, 20 or 30 min. (B) Cells were incubated for 15 min after the addition of 0, 50, 100, 200 or 500 μ M peroxynitrite (PN). Cells were lysed, proteins (25 μ g) were resolved by electrophoresis, and phosphotyrosine immunoreactivity was measured as described in the Experimental section. Panel (B) is a lighter exposure than (A) to permit the distinction of individual bands after treatment with 500 μ M peroxynitrite. Results shown are representative of three individual experiments. The positions of molecular mass standards are shown (in kDa) at the right.

and 50 kDa proteins. This result suggests that one effect of peroxynitrite is to increase tyrosine kinase activity. These results do not rule out the possibility that peroxynitrite also inhibited phosphotyrosine phosphatases, but this seems less likely considering the decreased phosphotyrosine immunoreactivity evident 20–30 min after exposure to peroxynitrite.

Immunoprecipitation methods were used to identify some of the phosphotyrosine proteins that were affected by peroxynitrite. Because phosphotyrosine proteins migrating near 120 kDa seemed to be both increased and decreased after treatment with peroxynitrite, we examined the tyrosine phosphorylation of two prominent phosphotyrosine proteins that migrate in this range: p120, a phosphotyrosine substrate of pp60^{src}, and focal adhesion

Figure 6 Effect of peroxynitrite on PAO-induced tyrosine phosphorylation

SH-SY5Y cells were untreated, incubated with 20 μ M PAO for 10 min, or preincubated with 200 μ M peroxynitrite (PN) for 5 min and then incubated with 20 μ M PAO for 10 min. Cells were lysed, proteins (25 μ g) were resolved by electrophoresis and phosphotyrosine immunoreactivity was measured as described in the Experimental section. Results shown are representative of three individual experiments. The positions of molecular mass standards are shown (in kDa) at the right.

Figure 7 Peroxynitrite transiently increased the tyrosine phosphorylation of p120

SH-SY5Y (A) or 1321N1 (B) cells were not treated (control) or treated with 200 μ M BSO for 48 h to deplete glutathione; the cells were then exposed to 0, 200 or 500 μ M peroxynitrite (PN) for 0, 5 or 10 min, and whole cell lysates were prepared. p120 was immunoprecipitated (IP) with anti-p120 antibody and the immunoprecipitate from 0.3 mg of cell protein was immunoblotted for phosphotyrosine (PY) or for p120 (results not shown; no changes in p120 levels were observed). The blots shown from each cell type are from a single experiment with a single gel, and are representative of three such experiments.

kinase (125 kDa). p120 was immunoprecipitated from SH-SY5Y cells to determine the time dependence, the peroxynitrite concentration dependence and the influence of glutathione depletion on p120 tyrosine phosphorylation. As shown in Figure 7(A) p120

Figure 8 Peroxynitrite decreased the tyrosine phosphorylation of (A) focal adhesion kinase (FAK) and (B) paxillin

SH-SY5Y cells were not treated (control) or treated with 200 μ M BSO for 48 h to deplete glutathione; the cells were then exposed to 0, 200 or 500 μ M peroxynitrite (PN) for 0, 5 or 10 min, and whole cell lysates were prepared. FAK and paxillin were immunoprecipitated (IP), and phosphotyrosine (PY) immunoreactivity and protein levels (results not shown, no changes were observed) were measured in the immunoprecipitate from 0.3 mg of cell protein. The blots shown from each cell type are from a single experiment with a single gel, and are representative of two such experiments.

tyrosine phosphorylation was increased 5 min after treatment with peroxynitrite, the increase was greater with 500 μ M (454 % of control) than 200 μ M (308 % of control) peroxynitrite, and there was a rapid reversal resulting in p120 phosphotyrosine immunoreactivity similar to controls within 10 min after exposure to peroxynitrite. In cells that had been previously depleted of glutathione by treatment with BSO, greater increases in p120 tyrosine phosphorylation were induced by both 200 μ M (508 % of control) and 500 μ M (992 % of control) peroxynitrite and, especially with 500 μ M peroxynitrite, the increase was prolonged. The level of p120 was not altered by any of these treatments (results not shown). Thus peroxynitrite induced a rapid and reversible increase in p120 tyrosine phosphorylation, the magnitude of which was influenced by the concentrations of both peroxynitrite and glutathione.

These effects of peroxynitrite on p120 tyrosine phosphorylation in SH-SY5Y cells were compared with effects in 1321N1 astrocytoma cells. There was no stimulation of p120 tyrosine phosphorylation by 200 μ M peroxynitrite in 1321N1 cells, even after the depletion of glutathione by treatment with BSO (Figure 7B). p120 phosphotyrosine immunoreactivity in 1321N1 cells was increased by 500 μ M peroxynitrite (513 % of control at 5 min) but there was no enhancement with glutathione depletion (488 % of control at 5 min). Thus the effect of peroxynitrite on p120 tyrosine phosphorylation, as well as on phosphoinositide hydrolysis, was less in 1321N1 cells than in SH-SY5Y cells, and this difference was retained after glutathione depletion.

In contrast with p120, only decreases in the phosphotyrosine immunoreactivity associated with immunoprecipitated focal adhesion kinase were detected after peroxynitrite treatment of SH-SY5Y cells (Figure 8A), e.g. a 50 % decrease occurred 5 min after

the addition of 200 μM peroxynitrite, whereas the level of focal adhesion kinase was unaffected by peroxynitrite (results not shown). Paxillin tyrosine phosphorylation often parallels that of focal adhesion kinase, and immunoprecipitation of paxillin demonstrated a marked decrease in paxillin phosphotyrosine immunoreactivity after the incubation of cells with 200 μM (40 % decrease after 5 min) or 500 μM (69 % decrease after 5 min) peroxynitrite (Figure 8B).

DISCUSSION

Peroxynitrite seems to contribute to the neuronal dysfunction associated with several neurodegenerative disorders, but little is known about which neuronal signalling systems mediate responses to peroxynitrite or are modulated by peroxynitrite. As with other biological oxidants [14], it seems likely that peroxynitrite modulates specific signalling systems, contributing to cell signalling processes as well as initiating neurotoxic effects. Because peroxynitrite is a strong biological oxidant, we hypothesized that peroxynitrite affects signalling systems that are modulated by other oxidants, such as the impairment of phosphoinositide signalling [16] and alterations in protein tyrosine phosphorylation [15] that are caused by H_2O_2 . Examination of these processes in human neuroblastoma SH-SY5Y cells demonstrated that peroxynitrite inhibited the activation of phosphoinositide signalling and both increased and decreased protein tyrosine phosphorylation.

The phosphoinositide signal transduction system is one of the major second-messenger systems in the brain that regulates protein phosphorylation, Ca^{2+} ion concentrations and ultimately gene expression through the modulation of transcription factor activation [30]. The phosphoinositide system consists of three primary proteins: a receptor, a G-protein and phospholipase C. Exposure of SH-SY5Y cells to relatively low concentrations of peroxynitrite significantly inhibited carbachol-induced phosphoinositide hydrolysis, a response mediated by M3 muscarinic receptors in these cells. Peroxynitrite only slightly inhibited phosphoinositide hydrolysis stimulated by NaF, which directly activates G-proteins, and did not inhibit phospholipase C activation by the Ca^{2+} ionophore ionomycin. These results indicate that receptor activation leading to stimulation of the G-proteins constitutes the target of peroxynitrite. Muscarinic receptor-stimulated phosphoinositide hydrolysis is severely impaired in Alzheimer's disease [31,32]. Because peroxynitrite has been implicated in the pathophysiology of Alzheimer's disease, in part from the identification of increased nitrotyrosine immunoreactivity in the post-mortem brain from subjects with Alzheimer's disease [12,13], it is apparent that both increases in peroxynitrite and decreases in phosphoinositide signalling coexist in Alzheimer's disease. Thus peroxynitrite might contribute to the impaired phosphoinositide signalling that occurs in Alzheimer's disease. However, impairment of phosphoinositide signalling is not a unique feature of peroxynitrite because H_2O_2 also inhibits phosphoinositide signalling in SH-SY5Y cells [16]. Thus impaired phosphoinositide signalling might result from the excessive production of a variety of reactive oxygen species in Alzheimer's disease and other neurodegenerative disorders. One consequence of impaired phosphoinositide signalling is a decreased activation of downstream transcription factors, such as AP-1 [16]. Thus inhibition of phosphoinositide signalling by peroxynitrite is likely to impact on transcription factor activation and consequently to alter the repertoire of genes expressed in affected cells, an action that could contribute to cell survival or cell death after challenge with peroxynitrite.

The greater susceptibility of phosphoinositide signalling to

inhibition by peroxynitrite in neuroblastoma than astrocytoma cells complements previous reports of differences in the susceptibilities of primary cultures of neurons and astrocytes to peroxynitrite [21–23]. It was suggested that this difference in vulnerability might be due primarily to the higher concentration of glutathione in astrocytes than neurons (reviewed in [23]). Although we confirmed that glutathione concentrations were higher in 1321N1 astrocytes than in SH-SY5Y neuroblastoma cells, even after approx. 99 % depletion of glutathione phosphoinositide signalling in astrocytoma cells was markedly less susceptible to inhibition by peroxynitrite than in neuroblastoma cells. Thus our findings support the conclusion that astrocytes are more resistant than neurons to the deleterious effects of peroxynitrite [21–23], but it seems that this difference regarding phosphoinositide signalling is due to cellular characteristics other than glutathione concentrations. Although the basis for this cell-type differential vulnerability to peroxynitrite is not known, it supports the contention that astrocytes might be a source of peroxynitrite production that is neurotoxic and that might contribute to selective neurodegeneration. Furthermore the markedly increased susceptibility to impairment by peroxynitrite of SH-SY5Y cells in which glutathione was depleted suggests that cellular variations in glutathione (or other antioxidant) concentrations might form a basis for the cell-selective neurodegeneration observed in Alzheimer's disease as well as other conditions, as has been suggested for Parkinson's disease [33,34].

Protein tyrosine phosphorylation, as well as protein nitrotyrosine formation, was also modified by peroxynitrite in SH-SY5Y cells. Although the identities of most phosphotyrosine proteins affected by peroxynitrite remain to be identified, peroxynitrite markedly increased the tyrosine phosphorylation of many proteins in a concentration-dependent manner. However, as was recently reported [18], increased phosphotyrosine immunoreactivity in human platelets was a transient response to peroxynitrite and there was a longer-lasting decrease in protein phosphotyrosine content. This transient increase contrasts distinctly with the apparently irreversible induction of protein nitrotyrosine immunoreactivity that followed exposure to peroxynitrite. Although investigators have previously speculated that protein tyrosine nitration and phosphorylation might be mutually exclusive, on the basis of experiments *in vitro* [35,36], the present experiments did not directly address that question. However, the simultaneous initial increases in tyrosine nitration and phosphorylation after exposure to peroxynitrite do not lend support to the mutually exclusive hypothesis, although site-specific measurements are clearly needed to resolve this issue. The temporal secondary decrease in overall protein phosphotyrosine immunoreactivity after peroxynitrite treatment seemed not to be related to tyrosine nitration because the latter remained unchanged with prolonged incubation times. Additionally, the marked increases in phosphotyrosine and nitrotyrosine immunoreactivities observed after the exposure of SH-SY5Y cells to 500 μM peroxynitrite, compared with 200 μM peroxynitrite, might be associated with the early stages of apoptosis induced by high concentrations of peroxynitrite [20].

The ramifications on cell function of the changes in protein tyrosine phosphorylation induced by 200 μM peroxynitrite cannot yet be specified because this response was biphasic and most of the proteins affected remain unidentified. However, protein tyrosine phosphorylation is a likely candidate for mediating cellular responses to peroxynitrite because this was responsive to relatively low concentrations of peroxynitrite and it was rapid and reversible; this also seems to be important in signalling induced by other biological oxidants. The decreased phosphotyrosine immunoreactivity of focal adhesion kinase and paxillin

after peroxynitrite treatment provides the identity of one signalling complex that is sensitive to peroxynitrite. The functions of these two adhesion-related proteins are still not completely described, but it is clear that they are important in the integrin and other signalling processes [37]. Such a large decrease in tyrosine phosphorylation of focal adhesion kinase and paxillin as occurred after exposure to peroxynitrite is therefore likely to impair significantly responses to such stimulants. The increased phosphotyrosine immunoreactivity of p120, a Src substrate, that was induced by peroxynitrite suggests that further studies of the effects of peroxynitrite on Src family kinase activities are warranted.

In summary, peroxynitrite was found to have three types of effect in human neuroblastoma SH-SY5Y cells. Peroxynitrite (1) increased protein tyrosine nitration, a modification that was long-lasting and was mostly restricted to cytosolic proteins, (2) inhibited the activation of the phosphoinositide signal transduction system, and (3) transiently increased, and also decreased, the phosphotyrosine content of several proteins. Each of these actions might contribute to peroxynitrite's effects on cell signalling, as well as to the deleterious effects of excessive peroxynitrite on neuronal function. These results raise critical topics to be addressed, including the identification of the key proteins affected by peroxynitrite among the numerous proteins that were modified, the roles of these actions of peroxynitrite in cellular survival and termination responses, and the relationships of these effects to the potential role of peroxynitrite in neurodegenerative diseases.

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