

Inhibition of PTEN by peroxynitrite activates the phosphoinositide-3-kinase/Akt neuroprotective signaling pathway

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Abstract

Peroxynitrite is usually considered as a neurotoxic nitric oxide-derivative. However, an increasing body of evidence suggests that, at low concentrations, peroxynitrite affords transient cytoprotection, both *in vitro* and *in vivo*. Here, we addressed the signaling mechanism responsible for this effect, and found that rat cortical neurons in primary culture acutely exposed to peroxynitrite (0.1 mmol/L) rapidly elicited Akt-Ser⁴⁷³ phosphorylation. Inhibition of phosphoinositide-3-kinase (PI3K)/Akt pathway with wortmannin or Akt small hairpin RNA (shRNA) abolished the ability of peroxynitrite to prevent etoposide-induced apoptotic death. Endogenous peroxynitrite formation by short-term incubation of neurons with glutamate stimulated Akt-Ser⁴⁷³ phosphorylation, whereas Akt shRNA enhanced the vulnerability of neurons against glutamate. We further show that Akt-Ser⁴⁷³ phosphorylation was consequence of the

oxidizing, but not the nitrating properties of peroxynitrite. Peroxynitrite failed to nitrate or phosphorylate neurotrophin tyrosine kinase receptors (Trks), and it did not modify the ability of brain-derived neurotrophic factor (BDNF), to phosphorylate its cognate receptor, TrkB; however, peroxynitrite enhanced BDNF-mediated Akt-Ser⁴⁷³ phosphorylation. Finally, we found that peroxynitrite-stimulated Akt-Ser⁴⁷³ phosphorylation was associated with an increased proportion of oxidized phosphoinositide phosphatase, PTEN, in neurons. Moreover, peroxynitrite prevented the increase of apoptotic neuronal death caused by over-expression of PTEN. Thus, peroxynitrite exerts neuroprotection by inhibiting PTEN, hence activating the anti-apoptotic PI3K/Akt pathway in primary neurons.

Keywords: Akt, anti-apoptotic, neuroprotection, peroxynitrite, PI3K, PTEN.

J. Neurochem. (2007) 10.1111/j.1471-4159.2007.04450.x

Peroxynitrite (PN), the product of the reaction between nitric oxide (NO) and superoxide (O₂^{•-}) (Blough and Zafriou 1985), is spontaneously formed in mammalian cells under physiological conditions (Radi *et al.* 2001; Pospel *et al.* 2002). Initial work addressing the pathophysiology of PN yielded the widely accepted notion that this compound would be the long-term cytotoxic NO-derivative (Beckman *et al.* 1990; Radi *et al.* 1991). Peroxynitrite involvement in cytotoxicity is documented by detecting protein 3-nitrotyrosination, i.e. the 'footprinting' like evidence for *in situ* PN formation (Ischiropoulos *et al.* 1992; Greenacre and Ischiropoulos 2001; Beal 2002), associated with neurodegeneration and other disorders (Bolaños *et al.* 1997; Lipton 1999; Guix *et al.* 2005). However, in apparent contradiction with

Received November 8, 2006; revised manuscript received January 5, 2007; accepted January 9, 2007.

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Abbreviations used: 7-AAD, 7-amino-actinomycin D; Akt, protein kinase B; APC, allophycocyanin; BDNF, brain-derived neurotrophic factor; CAT, catalase; DAPI, 4'-6-diamidino-2-phenylindole; GSH, glutathione ethyl ester; GFP, green fluorescent protein; Luc, luciferase; RNAi, RNA interference; PI3K, phosphoinositide-3-kinase; PFK-1, 6-phosphofructo-1-kinase; PN, peroxynitrite; PTP, protein tyrosine phosphatase; PTEN, phosphatase with tensin homology; shRNA, small hairpin RNA; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; Trk, neurotrophin tyrosine kinase receptor.

this notion, it has been shown that contractile failure (Lefer *et al.* 1997), arrhythmias (Altug *et al.* 1999) or necrosis (Nossuli *et al.* 1997, 1998) provoked by ischemia-reperfusion in the rat heart *in vivo* can be abolished by infusion with PN. Moreover, we previously showed that pre-treatment of primary neurons in culture with PN exerts transient neuroprotection against acute oxidative stress (Garcia Nogales *et al.* 2003).

These lines of evidence have raised the question of whether PN plays an active role in the etiology of neurodegenerative diseases, or its formation would be an emergency signal aimed at avoiding cellular death during oxidative insults (Ferdinandy and Schulz 2003; Bolaños *et al.* 2004). In fact, PN rapidly stimulates, in neurons, the activity of glucose-6-phosphate dehydrogenase (Garcia Nogales *et al.* 2003), the enzyme catalyzing the rate-limiting step of the pentose-phosphate pathway. Neurons can thus improve the capacity to regenerate glutathione (Garcia Nogales *et al.* 2003) – the antioxidant whose deficiency contributes to mitochondrial damage and apoptosis associated with certain neurodegenerative diseases (Bolaños *et al.* 1996; Jha *et al.* 2000; Diaz-Hernandez *et al.* 2005). Whilst such an antioxidant defense mechanism renders neurons more resistant to early cellular oxidation, it remains unclear whether PN would stimulate long-lasting signaling pathway(s) aimed at avoiding the propagation of neurodegeneration.

Previous work performed in other laboratories using skin fibroblasts has reported that PN can cooperate or interfere with tyrosine kinase receptor-mediated signaling pathways (Klotz *et al.* 2002; Söylemez *et al.* 2003). Peroxynitrite has been shown to oxidize – and thus inhibit – cysteinyl residue(s) of the protein tyrosine phosphatase (PTP) (Mallozzi *et al.* 1997; Takakura *et al.* 1999), leading to inhibition of tyrosine kinase receptor dephosphorylation (Mallozzi *et al.* 1997; Klotz *et al.* 2002). However, pre-treatment of skin fibroblast with PN, far from stimulating, attenuated PDGF-induced protein kinase B (Akt) phosphorylation (Klotz *et al.* 2000). Thus, the results obtained so far appear rather confusing (Bolaños *et al.* 2004), a fact that has prevented researchers from postulating a valid signaling mechanism that would be responsible for the neuroprotection afforded by PN.

Here, we aimed to investigate the involvement of the anti-apoptotic phosphoinositide-3-kinase (PI3K)/Akt signaling pathway in PN-mediated neuroprotection in rat cortical primary neurons. We demonstrate that PN affords neuroprotection by promoting Akt-Ser⁴⁷³ phosphorylation. Furthermore, we show that this effect is due to inhibition – by oxidation – of 3-phosphoinositide 3-phosphatase with tensin homology (PTEN), but not to modification of Trk receptors. These results suggest that caution should be taken when considering PN scavengers as neuroprotectants, and highlight the potential benefits of PTEN interference against neurodegeneration.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), poly-D-lysine, horse serum, cytosine arabinoside, (–)epicatechin, etoposide, *N*-ethylmaleimide (NEM), hydrogen peroxide, glutathione ethyl ester (GSH), glutamate, and glycine were obtained from Sigma Chemical Co. (St Louis, MO, USA). DMEM was supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL) (Sigma), except where indicated. Fetal bovine serum (FBS) was purchased from Roche Diagnostics (Heidelberg, Germany). Brain-derived neurotrophic factor (BDNF) was obtained from Peprotech EC (London, UK). Hybond[®] nitrocellulose membranes were from Amersham Biosciences (Buckinghamshire, UK). Plastic tissue culture dishes were purchased from Nunc (Roskilde, Denmark). Lipofectamine-2000 was purchased from Invitrogen (Groningen, The Netherlands). 2',7'-Dichlorodihydrofluorescein diacetate (H₂DCFDA) was obtained from Molecular Probes (Invitrogen). The PN donor 3-morpholino-synonimine (SIN-1) was purchased from Alexis Corp. (San Diego, CA, USA). Other substrates, inhibitors, enzymes, and coenzymes were purchased from Sigma, Roche Diagnostics, Promega Biotech (Madrid, Spain), Pharmacia or Merck (Darmstadt, Germany).

Design of shRNAs for Akt1/2

Akt1/2 knockdown was achieved by RNA interference using a vector-based shRNA approach (Brummelkamp *et al.* 2002). The shRNA-targeted cDNA sequence was chosen to knockdown Akt1 and Akt2 from either human and rat. The selected sequence was 5'-GTGGTCATGTACGAGATGA-3' [corresponding to nt. 1207–1225 (NM_005163, human Akt1), 1215–1233 (NM_001626, human Akt2), 1051–1069 (NM_033230, rat Akt1) and 1293–1311 (NM_017093, rat Akt2)], which was designed according to a previously reported rational protocol (Reynolds *et al.* 2004) to accomplish criteria I, II, III, IV, VI, VII and VIII. As controls, we used either the firefly luciferase-targeted oligonucleotide 5'-CTGACGCGGAATACTTCGA-3', as previously reported (Ohtsuka *et al.* 2004) or a scrambled Akt sequence (5'-CGTTAC-ATCACGATCCTAC-3'). All sequences were BLAST-confirmed for specificity. The forward and reverse synthetic 64-nt oligonucleotides (Isogen Life Technologies, Maarsen, The Netherlands) were designed, annealed, and inserted into the *BgIII/HindIII* sites of the pSuper-neo/green fluorescent protein (GFP) vector, following the manufacturer's instructions (Oligoengine, Seattle, WA, USA). These constructions express 19 base-pair 9-nt stem-loop shRNAs targeted against human and rat Akt1/2 (Akt shRNA), luciferase (used as a shRNA control) or scrambled Akt (used as another shRNA control) mRNAs. The concomitant expression of GFP from this vector allowed the identification of transfected cells by flow cytometry.

Plasmid constructions

The cDNAs encoding wild type and mutated (Cys¹²⁴ → Ser¹²⁴) PTEN were generous gifts from Prof. R. Pulido (Centro de Investigación Príncipe Felipe, Valencia, Spain). Both cDNAs were subcloned into the corresponding *EcoRI* or *BamHI* sites of the pIRES2-EGFP mammalian expression vector (Invitrogen) and sequenced for confirmation. The concomitant expression of GFP

from this vector allowed the identification of transfected cells by flow cytometry.

Neurons in primary culture

Cerebral cortex neurons in primary culture were prepared from fetal Wistar rats at 16 days of gestation (Almeida *et al.* 2004). Dissociated cell suspensions were plated at a density of 2.5×10^5 cells/cm² in 60 cm² Petri dishes, or 6- or 12-wells plates, previously coated with poly-D-lysine (15 µg/mL) in DMEM supplemented with 10% fetal calf serum. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% air. Forty-eight hours after plating, the medium was replaced with DMEM supplemented with 5% horse serum, 20 mmol/L D-glucose and cytosine arabinoside (10 µmol/L) to prevent non-neuronal proliferation. Neurons, which were approximately 99% Map2-positive, were transfected on day 4 (for Akt knockdown) or 6 (for PTEN expression).

HEK293T cells

Human embryonic kidney 293T (HEK293T) cells were maintained in DMEM supplemented with 10% (v/v) fetal calf serum. Cells were re-seeded at 10^5 cells/cm² 1 day before transfections.

Synthesis of peroxyntirite

Peroxyntirite was synthesized and quantified spectrophotometrically ($\epsilon_{302} = 1670$ /M/cm) as previously described (Hughes and Nicklin 1968). Alkaline stock solutions, with an approximate PN concentration of 0.5 mol/L, were stable at -70°C for at least 3–4 months.

Cell treatments

Cell transfections were performed with pSuper-neo/gfp- or pIRES2-EGFP-derived plasmid constructions using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. After 6 h, the medium was removed and cells were further incubated for the indicated time periods in complete culture medium. Peroxyntirite was added to neurons in *bolis* of 50, 100 or 500 µmol/L (final concentration) from 500-fold concentrated stock solutions in buffered Hanks' solution (5.26 mmol/L KCl, 0.43 mmol/L KH₂H₂PO₄, 132.4 mmol/L NaCl, 4.09 mmol/L NaHCO₃, 0.33 mmol/L Na₂HPO₄, 20 mmol/L glucose, 2 mmol/L CaCl₂, and 20 mmol/L HEPES, final pH 7.4); the corresponding control incubations consisted of previously degraded (for 3 h at 37°C) PN solutions. Owing to the relative instability of authentic PN solutions at physiological pH, neurons were also incubated, for 15 min, in DMEM containing SIN-1, a PN source formed from nitric oxide and superoxide (Feelisch *et al.* 1989; Schrammel *et al.* 1998). Preliminary experiments using a NO-sensitive electrode (ISO-NO) (not shown) suggested an approximate PN formation from SIN-1 (0.5 mmol/L) of about 10 µmol/L, as previously reported (Schrammel *et al.* 1998). To ensure immediate maximum PN release from SIN-1, all SIN-1-containing solutions were always pre-incubated in DMEM at 37°C for 20 min before the addition to the cells. SIN-1 controls experiments were performed using a degraded SIN-1 solution (72 h at 37°C). To activate glutamate receptors thus promoting the endogenous formation of PN (Almeida and Bolaños 2001), neurons were incubated, for 5 min, with 100 µmol/L glutamate (plus 10 µmol/L glycine) in buffered Hanks' solution (pH 7.4), either in the absence or in the presence of wortmannin (100 nmol/L), GSH (5 mmol/L) or superoxide dismutase (SOD)

plus catalase (CAT) (100 U/mL each). Neurons were then washed and further incubated in culture medium for the indicated time period. To ensure a low degree of phosphorylation of Trk receptor and Akt in the controls, all experiments were carried out in neurons pre-incubated in serum-free DMEM for 6 h. Experiments combining PN and BDNF were performed as follows: neurons were incubated (in buffered Hanks' solution) for 5 min with PN (100 µmol/L, or its degraded solution as control) followed by a further 5 min incubation in the absence or presence of BDNF (50 ng/mL); alternatively, neurons were incubated for 5 min in the absence or presence of BDNF (50 ng/mL) followed by a further 5 min incubation with PN (100 µmol/L or its degraded solution as control).

Western blotting

Cells were lysed for 20 min at 4°C in a buffer containing 1% nonidet NP-40, 5 mmol/L EDTA, 20 mmol/L Tris, pH 8.0, 137 mmol/L NaCl, 10% glycerol, 100 µmol/L phenylmethylsulfonyl fluoride, 50 µg/mL anti-papain, 50 µg/mL pepstatin, 50 µg/mL amastatin, 50 µg/mL leupeptine, 50 µg/mL bestatin, 1 mmol/L *o*-vanadate, and 50 µg/mL soybean trypsin inhibitor. Extracts were centrifuged at 13 000 g for 20 min at 4°C and aliquots containing 100 µg of protein from each sample, as determined extemporarily following the BCATM Protein assay kit (Pierce, Rockford, IL, USA) using albumin as standard and the BenchMarkTM pre-stained protein ladder (Invitrogen), and were subjected to sodium dodecyl sulfate (SDS, 0.1%)-polyacrylamide gel electrophoresis (PAGE) on a 7% (Trk receptors and 3-nitrotyrosine), 8% (Akt, phospho-Akt) or 10% (PTEN) acrylamide gel (MiniProtean[®]; Bio-Rad, Hercules, CA, USA). The resolved proteins were transferred electrophoretically to nitrocellulose membranes (Hybond-ECL; Amersham Bioscience Europe GmbH, Barcelona, Spain). Membranes were blocked with 5% (w/v) low-fat milk in 20 mmol/L Tris, 500 mmol/L NaCl, 0.1% (w/v) Tween-20 (pH 7.5) for 1 h. Immunoblotting was performed using α -panTrk antibody (Martin-Zanca *et al.* 1989), α -phospho-Tyr⁴⁹⁰-Trk (Cell Signaling Technology), α -Akt (Cell Signaling Technology), α -phospho-Ser⁴⁷³-Akt (α -pAkt; Cell Signaling Technology), α -PTEN (421B, a generous gift from Prof. R. Pulido (Centro de Investigación Príncipe Felipe, Valencia, Spain) or α -3-nitro-Tyr (a generous gift from Prof. J. Rodrigo, Instituto Cajal, Madrid, Spain) antibodies. Anti-phosphofruktokinase-1 (PFK1) was used as loading control (Almeida *et al.* 2004). Anti-rabbit secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were incubated with horseradish peroxidase-conjugated goat antirabbit IgG (1 : 10 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and immediately incubated with Supersignal West Dura (Pierce) for 5 min before exposed to Kodak XAR-5 film (Sigma) for 1–3 min. Autoradiograms were scanned.

Identification of reduced and oxidized forms of PTEN by immunoblot analysis

This was performed following the method of Lee *et al.* (2002). In brief, we followed identical protocol to that for western blotting, with the exceptions that the buffer contained 40 mmol/L NEM and, after centrifugation, aliquots were subjected to SDS-PAGE under reducing (715 mmol/L β -mercaptoethanol) or non-reducing (without β -mercaptoethanol) conditions.

Slot blotting

This was performed by vacuum-mediated transference of cellular protein extracts – obtained as indicated for western blotting – to nitrocellulose membranes, followed by immunoblotting against α -nitro-Tyr antibody, as indicated in the western blotting section.

Immunoprecipitation

Cells were lysed in a buffer containing 1% Nonidet NP-40, 137 mmol/L NaCl, 20 mmol/L Tris (pH 8.0), 10% glicerol, 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μ g/mL leupeptin, 5 μ g/mL aprotinin F. One milligram of protein was incubated with α -pan-Akt in the presence of 24 μ L of protein A-sepharose during 4 h at 4°C. The immunoprecipitated complex was extensively washed with the lysing buffer and the mixture was then subjected to SDS-PAGE.

Flow cytometric analysis of apoptotic cell death

Allophycocyanin (APC)-conjugated annexin-V and 7-amino-actinomycin D (7-AAD) (Apoptosis Assay Kit; Becton Dickinson Biosciences, San José, CA, USA) were used to quantitatively determine the percentage of apoptotic cells by flow cytometry. Cells were stained with annexin V-APC and 7-AAD, following the manufacturer's instructions, and were analysed on a FACScalibur flow cytometer (15 mW argon ion laser tuned at 488 nm; CellQuest software, Becton Dickinson Biosciences). Both GFP⁺ and GFP⁻ cells were analyzed separately, and the annexin V-APC-stained cells that were 7-AAD-negative were considered apoptotic (Almeida *et al.* 2004).

Analysis of apoptotic death by 4'-6-diamidino-2-phenylindole nuclear staining

Neurons were fixed with 4% [v/v, in phosphate-buffered saline (PBS)] paraformaldehyde for 30 min at 25°C, rinsed with PBS and incubated with 4'-6-diamidino-2-phenylindole (DAPI) (30 μ mol/L; Sigma). After 10 min, cells were washed three times with PBS and their nuclei examined under a fluorescence microscope by an author blinded to the test. A total of approximately 200 cells per condition in three different cultures were quantified, and the results were expressed as the percentage of condensed or fragmented nuclei.

NADPH determination

Neurons were washed with ice-cold PBS and lysed in 0.5 mol/L KOH in 50% (vol/vol) ethanol. NADPH determination was performed exactly as previously described (Garcia Nogales *et al.* 2003).

Immunofluorescence measurement of dichlorodihydrofluorescein (H₂DCFDA) oxidation

Neurons were incubated with 10 μ mol/L H₂DCFDA in Hanks' solution (pH 7.4). After 30 min at 37°C, cells were washed twice with PBS and incubated with PN for 5 min in the presence of varying concentrations of epicatechin (10, 100 or 1000 μ mol/L). Dichlorofluorescein fluorescence was measured in a Fluoroskan Ascent Fluorimeter (Thermo Electron Corporation, Milford, MA, USA; excitation at 485 nm, emission at 538 nm) and expressed in arbitrary units, as previously reported (Wang and Joseph 1999).

Statistical analysis

Measurements from individual cultures were always performed in triplicate. The results are expressed as mean \pm SEM values for three different culture preparations. Statistical analysis of the results was performed by one-way analysis of variance, followed by the least significant difference multiple range test. In all cases, $p < 0.05$ was considered significant.

Results

Peroxyinitrite prevents etoposide-mediated apoptotic death by stimulating the PI3K/Akt pathway in primary cortical neurons

In a previous study, we demonstrated that PN, applied either directly or released from the PN donor SIN-1, time-dependently protected neurons against the apoptotic death caused by acute oxidative stress (Garcia Nogales *et al.* 2003). Furthermore, we showed that protection was maximal at short-term periods (1–4 h), progressively disappearing thereafter (Garcia Nogales *et al.* 2003). To explore the possibility that such protection could be a consequence of the activation of the anti-apoptotic pathway, phosphoinositide-3-kinase (PI3K)/Akt, we first investigated whether Akt was phosphorylated by PN treatment. As shown in Fig. 1a (upper panel), PN rapidly (5 min) triggered Akt phosphorylation at Ser⁴⁷³ in a concentration-dependent manner, being evident at 100 μ mol/L. To test whether Akt-Ser⁴⁷³ phosphorylation was a consequence of PI3K activity, we used the PI3K inhibitor, wortmannin. As shown in Fig. 1a (lower panel), wortmannin (100 nmol/L) efficiently prevented Akt-Ser⁴⁷³ phosphorylation. Next, we confirmed the neuroprotective role of PN and showed that acute incubation of neurons with a bolus of PN at 100 μ mol/L, i.e. the concentration shown to promote Akt-Ser⁴⁷³ phosphorylation (Fig. 1a) protected them against the initiation of apoptotic death caused by etoposide (10 μ g/mL; 1 h) (Fig. 1b). Etoposide was used as the apoptotic death trigger, since this procedure has been previously validated to be suitable for the investigation of apoptotic death and neuroprotective pathways in cortical neurons (Nakajima *et al.* 1994). Our results thus suggest that both phenomena – Akt phosphorylation and neuroprotection – might be linked. Blockade of PI3K activity with wortmannin fully abolished the protection that PN afforded against etoposide-mediated apoptotic death (Fig. 1b). We further confirmed these results by determining the proportion of condensed or fragmented nuclei using DAPI staining (Figs 1c and d). As shown in Fig. 1d, the increase in apoptotic death triggered by etoposide as assessed with DAPI was slightly higher than that using annexin V (Fig. 1b), although PN afforded full protection (in the absence of wortmannin) regardless of the method used (Fig. 1d). We next explored whether Akt phosphorylation could be linked to the increased NADPH

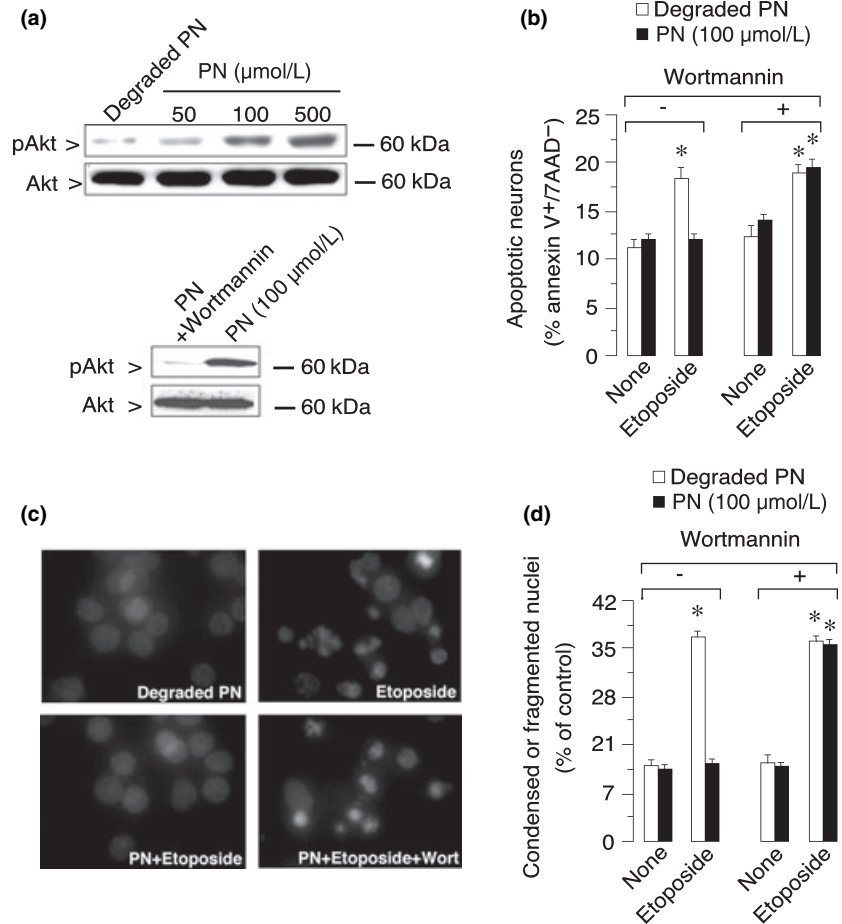


Fig. 1 Peroxynitrite prevents etoposide-mediated apoptotic death through phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) pathway in neurons in primary culture. (a) Incubation of neurons with peroxynitrite concentration-dependently promoted Akt phosphorylation (phospho-Ser⁴⁷³) after 5 min (upper panel), an effect that was prevented by wortmannin (100 nmol/L) (lower panel). (b) Pre-treatment of neurons with 100 μmol/L peroxynitrite (5 min) prevented apoptotic death promoted by etoposide (10 μg/mL) after 1 h, and effect that was abolished by wortmannin (100 nmol/L). (c) Typical microphotographs showing condensed or fragmented nuclei by 4'-6-diamidino-2-phenylindole staining under identical conditions as those used for (b). (d) Quantification of the results shown in (c). * $p < 0.05$ when compared with the corresponding control (degraded PN) values.

concentrations by PN that we previously reported (Garcia Nogales *et al.* 2003). We found that the increase in neuronal NADPH concentrations caused by PN was not impaired by wortmannin (in pmol/mg of protein, degraded PN, 52 ± 4 ; 100 μmol/L PN, 120 ± 11 ; degraded PN + wortmannin, 55 ± 6 ; 100 μmol/L PN + wortmannin, 121 ± 10 ; $n = 3$), ruling out the involvement of the PI3K/Akt pathway in this effect.

We next designed a vector-based small hairpin RNA (shRNA) strategy against Akt to more specifically knock-down the PI3K/Akt pathway. As shown in Fig. 2a, expression of a plasmid vector carrying the shRNA Akt sequence efficiently reduced Akt protein in HEK293T cells 2 days after transfection. Control transfections were carried out expressing either a shRNA sequence against luciferase (shRNA Luc) (Diaz-Hernandez *et al.* 2005) or a corresponding scrambled Akt sequence. Transfection of neurons in primary culture with the shRNA Akt only resulted in a low degree of loss of neuronal survival when compared with the shRNA Luc or scrambled Akt transfections, at least at the time when the experiments were performed (3 days after transfection), although the values did not reach statistical differences (Fig. 2b). Etoposide triggered a rapid (1 h)

apoptotic response, whereas PN did not. Furthermore, PN prevented etoposide-mediated apoptotic death in neurons transfected with the shRNA Luc or scrambled Akt, but not in those transfected with shRNA Akt (Fig. 2b). These results further support the essential role of Akt during PN neuroprotection. We next studied whether Akt silencing abolished neuroprotection by PN formed from SIN-1, a compound known to form PN by simultaneously releasing nitric oxide and superoxide. As shown in Fig. 2c, Akt-Ser⁴⁷³ was phosphorylated 15 min after incubation with SIN-1, an effect that was prevented by wortmannin. Furthermore, SIN-1 prevented etoposide-mediated apoptotic death in neurons transfected with shRNA Luc, but not in those transfected with shRNA Akt (Fig. 2d). Next, we incubated neurons with glutamate (100 μmol/L for 5 min), a treatment that we, and others, have shown to trigger spontaneous *in situ* PN formation (Almeida and Bolaños 2001; El-Remessy *et al.* 2003). We found that glutamate rapidly (5 min) increased Akt-Ser⁴⁷³ phosphorylation in primary neurons (Fig. 2e), confirming a previous work (Sutton and Chandler 2002). Furthermore, this effect was prevented with wortmannin (Fig. 2e), implicating the PI3K/Akt pathway in the PN-mediated Akt-Ser⁴⁷³ phosphorylation. Long-term (18–24 h)

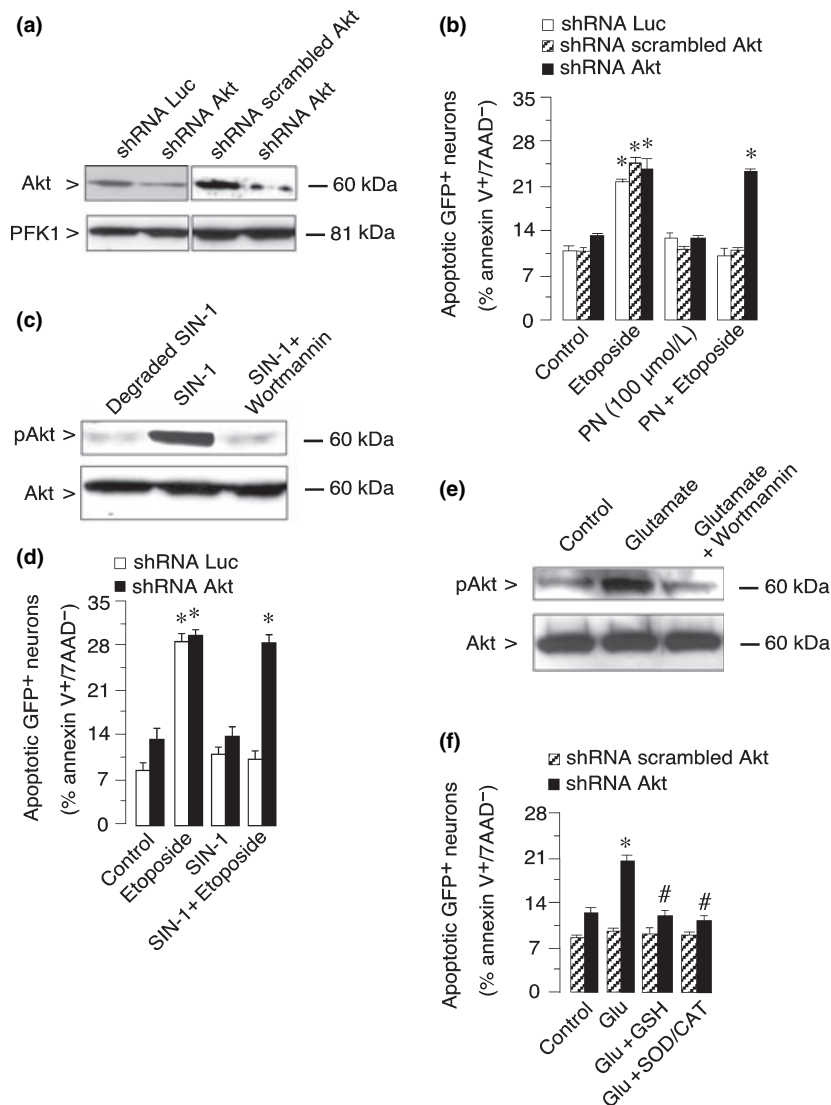


Fig. 2 Knockdown of protein kinase B (Akt) by small interfering RNA abolishes neuroprotection by peroxynitrite and triggers neurotoxicity by glutamate in neurons in primary culture. (a) To confirm the efficacy of the designed small hairpin RNA (shRNA), HEK293T cells were transfected with a pSuper-neo/gfp plasmid vector expressing an shRNA against Akt, which promoted Akt knockdown 2 days after transfections, when compared with appropriate controls (shRNA Luc or shRNA scrambled Akt), as evidenced by western blotting. (b) Transfection of neurons with shRNA Akt did not cause, after 3 days, apoptotic death when compared with controls (shRNA Luc or shRNA scrambled Akt); etoposide triggered apoptotic death in neurons regardless of Akt expression; incubation of transfected neurons with peroxynitrite, alone, is not neurotoxic; however, pre-incubation of neurons with peroxynitrite prevented etoposide-mediated apoptotic death in controls (shRNA Luc or shRNA scrambled Akt), but not in Akt-silenced (shRNA Akt) neurons. (c) Incubation of neurons with SIN-1

incubation of neurons after glutamate treatment often causes neurotoxicity (excitotoxicity) (Dawson *et al.* 1991; Almeida and Bolaños 2001). In contrast, acute glutamate treatment

(0.5 mmol/L) promoted Akt phosphorylation (phospho-Ser⁴⁷³) after 15 min, an effect that was prevented by wortmannin (100 nmol/L). (d) Incubation of neurons with SIN-1 (0.5 mmol/L), alone, is not neurotoxic; however, pre-incubation of neurons with SIN-1 (0.5 mmol/L) prevented etoposide-mediated apoptotic death in control (shRNA Luc), but not in Akt-silenced (shRNA Akt) neurons. (e) Incubation of neurons with glutamate (100 μmol/L, 5 min) triggered Akt phosphorylation, an effect that was prevented by wortmannin (100 nmol/L). (f) Akt knockdown by shRNA in neurons enhanced glutamate-mediated apoptotic death 1 h after treatment, an effect that was prevented by either glutathione ethyl ester (5 mmol/L) or superoxide dismutase plus CAT (100 U/mL each). **p* < 0.05 when compared with the corresponding control values. #*p* < 0.05 when compared with corresponding glutamate-treated shRNA Akt values. Glu, glutamate; GSH, glutathione ethyl ester; SOD/CAT, superoxide dismutase plus catalase.

(100 μmol/L for 5 min) did not result in short-term (1 h after glutamate challenge) neurotoxicity (Fig. 2f); however, Akt silencing with shRNA enhanced apoptotic death (Fig. 2f).

These results confirm the involvement of the PI3K/Akt neuroprotective signaling pathway in the short-term incubation of cortical neurons with glutamate. To ascertain whether PN was involved in glutamate-mediated neuroprotection, GSH – a PN scavenger – or SOD/CAT – superoxide and H₂O₂ scavengers – were used. The results (Fig. 2f) show that both treatments significantly prevented shRNA Akt-mediated apoptotic death in glutamate-treated neurons. In view of the difficulties to selectively scavenge PN without altering other potential oxidants within the cell, these results do not unambiguously determine the involvement of PN in glutamate-mediated short-term neuroprotection. However, these data clearly indicate that short-term glutamate treatment activates the PI3K/Akt pathway in primary cortical neurons, and that this activation is required for PN (and/or a related oxidant)-mediated neuroprotection.

The phosphorylation of Akt-Ser⁴⁷³ promoted by peroxynitrite is not mediated through phosphorylation or nitration of Trk receptors in primary neurons

Cortical neurons express the neurotrophin receptor TrkB, and are protected from apoptosis by its cognate ligand, BDNF (Hetman *et al.* 1999). To try to understand the mechanism responsible for PN-mediated Ser⁴⁷³-Akt phosphorylation, we first focused on Trk receptors, since their activation – by phosphorylation – can stimulate the PI3K/Akt pathway. Cortical neurons were incubated with PN, alone, or in combination with BDNF. As shown in Fig. 3a, BDNF triggered the expected response by rapidly (10 min) promoting the phosphorylation of Trk receptors at Tyr⁴⁹⁰, hence confirming the expression of functional TrkB in these cells. In addition, PN treatment did not elicit Trk phosphorylation (Fig. 3a). Moreover, pre-treatment of neurons with BDNF did not affect the response to PN; and, pre-treatment with PN did not affect the response to BDNF (Fig. 3a). In view that PN was unable to alter Trk phosphorylation, we next studied whether it modified Trk receptor nitration, since such modification has been suggested to occur, at least in other cellular systems (Bolaños *et al.* 2004). Indeed, PN caused an increase in overall 3-nitrotyrosination of cell proteins (Fig. 3b, left panel). To ascertain whether some of these proteins were Trk receptor(s), we performed immunoprecipitation using a pan-Trk antibody, and the precipitated proteins were subjected to western blotting with a nitro-tyrosine antibody. As shown in Fig. 3b (right panel), PN did not cause Trk receptor nitration, as judged by the lack of 3-nitrotyrosinated proteins in Trk immunoprecipitates. We next showed that PN and BDNF-induced Akt phosphorylation were additive. As shown in Fig. 3c, PN increased the degree of Ser⁴⁷³-Akt phosphorylation caused by BDNF, and BDNF increased the degree of Ser⁴⁷³-Akt phosphorylation caused by PN. These results confirm that PN triggers Akt phosphorylation through a mechanism that does not involve Trk receptors in primary neurons, and suggest that PN and BDNF

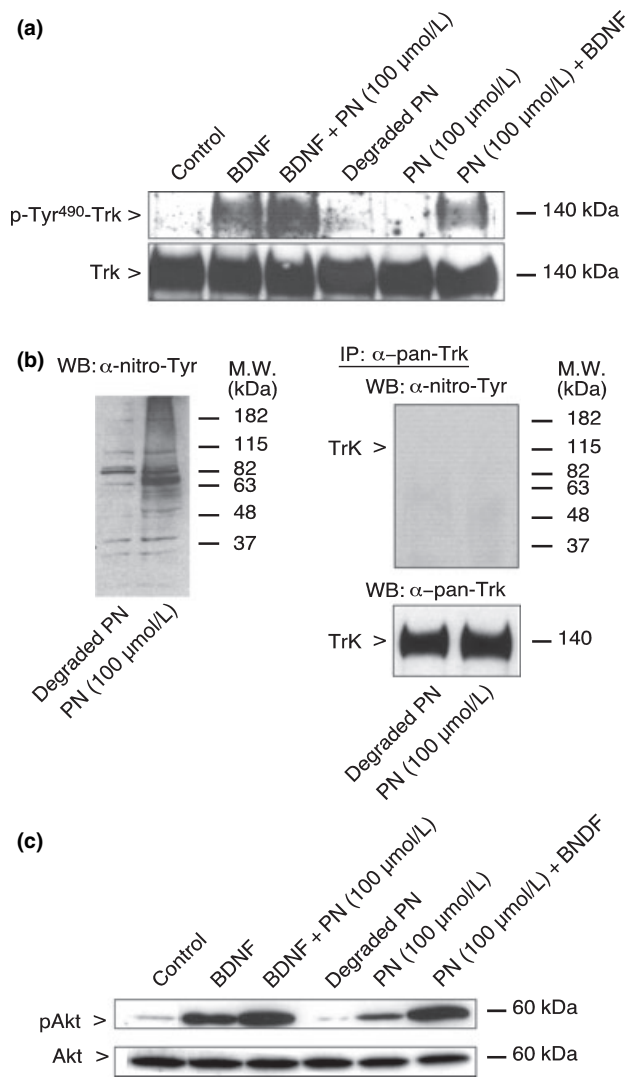


Fig. 3 Phosphorylation of protein kinase B (Akt) by peroxynitrite is not mediated through phosphorylation or nitration of Trk receptors in primary neurons. (a) Brain-derived neurotrophic factor (BDNF, 50 ng/mL), but not peroxynitrite, increased Trk Tyr⁴⁹⁰ phosphorylation after 5 min of incubation. (b) Peroxynitrite promoted overall protein nitrotyrosination (left panel) in neurons, but immunoprecipitation against Trk receptors revealed that these receptors were not nitrotyrosinated (right panel). (c) The phosphorylation of Akt-Ser⁴⁷³ by peroxynitrite and BDNF (50 ng/mL) was additive.

activate different signaling pathways that converge in Akt activation.

Protein oxidation, but not nitration, accounts for peroxynitrite-mediated neuroprotection and Akt phosphorylation

Previous studies have demonstrated that PN can alter protein functions by nitrating or oxidizing critical Tyr or Cys residues, respectively (Bolaños *et al.* 2004). Accordingly, we next aimed to investigate whether the

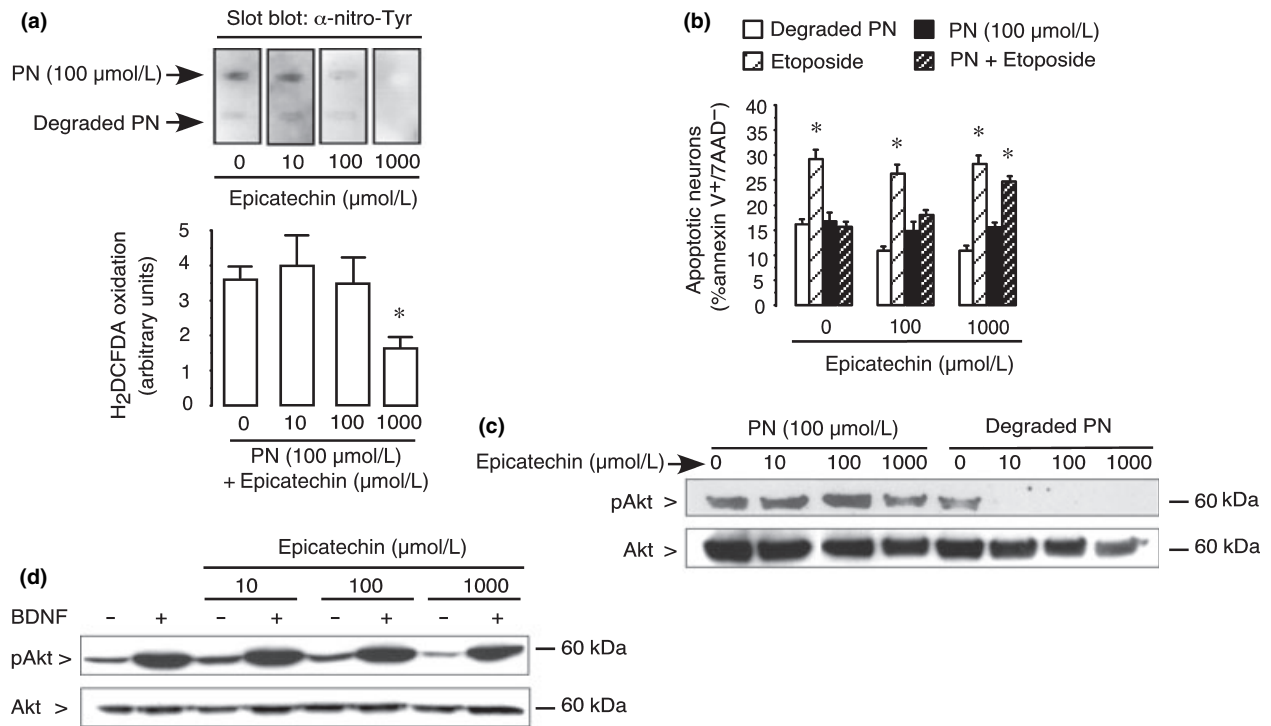


Fig. 4 Protein oxidation, but not nitration, accounts for the neuroprotective effect of peroxynitrite. (a) Epicatechin prevented both nitration (upper panel) and oxidation (lower panel) by peroxynitrite; however, at 100 μ mol/L, epicatechin prevented nitration, but not oxidation, whereas at 1000 μ mol/L it prevented both nitration and oxidation. (b) Peroxynitrite prevented the apoptosis caused by etoposide

at 100 μ mol/L, but not at 1000 μ mol/L, epicatechin. (c) Peroxynitrite phosphorylated Akt-Ser⁴⁷³ at 100 μ mol/L, but not at 1000 μ mol/L, epicatechin. (d) Epicatechin does not interfere with BDNF (50 ng/mL)-mediated Akt-Ser⁴⁷³ phosphorylation. * $p < 0.05$ when compared with the corresponding control (degraded PN) values.

phosphorylation of Akt was consequence of either the nitrating or the oxidizing effect of PN in primary neurons. This was performed with using (–)-epicatechin, an antioxidant compound known to differentially prevent nitration and oxidation by PN (Schroeder *et al.* 2001). To validate the efficacy of this compound under our experimental conditions, we incubated neurons with PN in the presence of increasing concentrations of epicatechin. Then, we determined the oxidation of the fluorescent probe H₂DCFDA and the presence of nitrated proteins in the same cells. As shown in Fig. 4a, 100 μ mol/L epicatechin prevented protein nitration (upper panel) without altering oxidation (lower panel) by PN; however, 1000 μ mol/L epicatechin prevented both nitration and oxidation. Using an identical experimental protocol, we then investigated whether epicatechin was able to alter the neuroprotective effect of PN. As shown in Fig. 4b, PN prevented etoposide-mediated apoptotic death at 100 μ mol/L, but not at 1000 μ mol/L, epicatechin. In addition, we found that PN phosphorylated Akt-Ser⁴⁷³ at 100 μ mol/L, but not at 1000 μ mol/L, epicatechin (Fig. 4c). We also observed that epicatechin decreased Akt-Ser⁴⁷³ phosphorylation in control (degraded PN-treated) neurons (Fig. 4c), suggesting that epicatechin might directly affect

the ability of Akt to be phosphorylated. To test this possibility, neurons were subjected to BDNF-mediated Akt-Ser⁴⁷³ phosphorylation in the presence of increasing concentrations of epicatechin. As depicted in Fig. 4d, epicatechin was unable to prevent BDNF-mediated Akt-Ser⁴⁷³ phosphorylation. Together, these results suggest that neuroprotection and Akt phosphorylation were due to the oxidizing, not nitrating, effect of PN.

Peroxynitrite oxidizes phosphoinositide phosphatase and prevents the excess apoptotic death caused by PTEN over-expression in primary neurons

In view that Akt phosphorylation and neuroprotection exerted by PN were not due to the upstream activation of the Trk-PI3K/Akt pathway, we wondered whether those effects were related to the impairment of Akt dephosphorylation. Akt is an anti-apoptotic kinase that is activated by phosphorylation, promoted by phosphatidylinositol-dependent kinase 1 (PDK1), a kinase that is in turn activated by the effector phosphatidyl-inositol-3,4,5-trisphosphate (PIP₃), i.e. the product of PI3K activity. PTEN is the phosphatase that eliminates the 3-phosphate of PIP₃, antagonizing PDK1-mediated Ser⁴⁷³-Akt phosphorylation. Conversely, PTEN

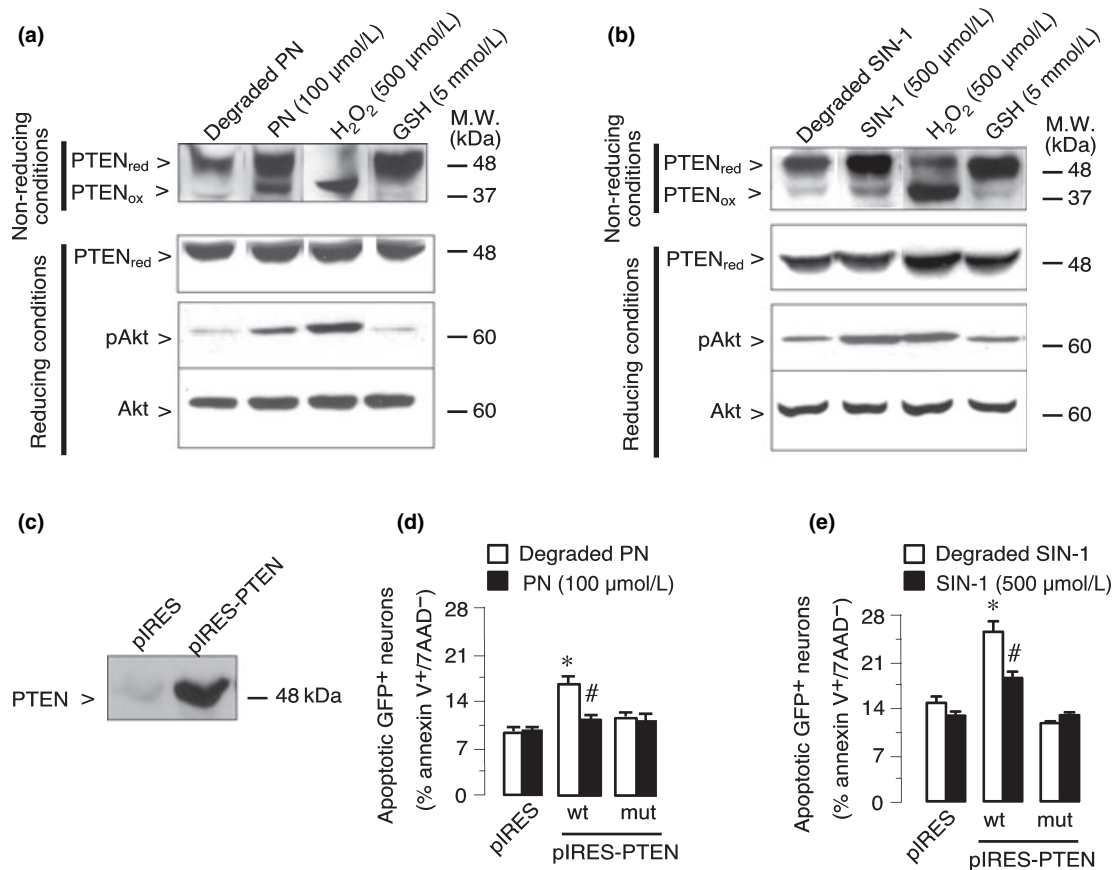


Fig. 5 Peroxynitrite oxidizes phosphatase with tensin homology (PTEN) and prevents apoptotic death caused by PTEN over-expression in primary neurons. (a) Incubation of neurons with peroxynitrite promoted the appearance of oxidized forms of PTEN, as judged by western blotting under non-reducing conditions. H₂O₂ and glutathione ethyl-ester (GSH) were used as controls for strong oxidizing- and strong-reducing conditions, respectively. Oxidation of PTEN was associated with Akt Ser⁴⁷³ phosphorylation. (b) PTEN oxidation by peroxynitrite was confirmed using SIN-1 (0.5 mmol/L; 15 min). (c) HEK293T cells were transfected with a cDNA encoding wild type

PTEN to confirm protein expression (upper panel). Transfection of neurons in primary culture with wild type (wt) PTEN significantly increased, 18 h later, apoptotic death when compared with transfections with either the empty vector (pIRES) or the mutated (mut, Cys¹²⁴ → Ser¹²⁴) form of PTEN. (d and e), This effect was prevented by incubating neurons with peroxynitrite (5 min, d) or SIN-1 (15 min, e) 1 h before apoptotic death assessments. **p* < 0.05 when compared with the empty vector (pIRES) or mutated PTEN values. #*p* < 0.05 when compared with the corresponding control (degraded PN) value.

inhibition, or mutation, impairs PIP₃ inactivation, thus enhancing the Akt-mediated anti-apoptotic pathway (Cully *et al.* 2006). To address the possible role of PN on this PI3K-PTEN network, we first investigated whether PN altered the redox status of PTEN, a mechanism reported to regulate its activity (Kwon *et al.* 2004). PTEN contains a critical Cys¹²⁴ residue that is essential for the correct protein folding and enzyme activity (Lee *et al.* 2002; Kwon *et al.* 2004). Oxidation of PTEN hence inhibits its activity. To ascertain whether PN altered the redox status of PTEN, we performed western blotting under non-reducing conditions in protein extracts obtained from PN-treated primary neurons. As shown in Figs 5a and b, PN or SIN-1 rapidly (5 or 15 min, respectively) oxidized PTEN along with Akt phosphorylation. Furthermore, H₂O₂, which is well known to promote

PTEN oxidation (Lee *et al.* 2002) (Figs 5a and b), also triggered Akt phosphorylation in primary neurons. These results suggest that oxidation of PTEN by PN can account for the rapid increase of Akt-Ser⁴⁷³ phosphorylation. Finally, we aimed to elucidate whether PTEN oxidation by PN was responsible for its neuroprotective properties. To accomplish this, we over-expressed the full-length PTEN cDNA (Fig. 5c), or its inactive form carrying a Cys¹²⁴ → Ser¹²⁴ mutation in cortical neurons. As shown in Fig. 5d, transfection of primary neurons with wild type PTEN spontaneously (after 18 h) elicited apoptotic death; however, transfection of neurons with the mutated form did not elicit apoptotic death when compared with neurons expressing the empty vector (pIRES) (Figs 5d and e). Interestingly, incubation of neurons with PN or SIN-1 (5 or 15 min, respectively),

1 h before apoptotic death assessments, prevented the apoptotic death caused by over-expression of the wild type PTEN, but did not alter the response to expression of the mutated form (Figs 5d and e).

Discussion

Here we show that incubation of primary cortical neurons in culture with PN – either authentic or formed from nitric oxide and superoxide – rapidly triggers phosphorylation of Akt at Ser⁴⁷³ and protects neurons against the apoptotic death caused by etoposide. Such effects were abolished by inhibition of PI3K activity with wortmannin, or by Akt shRNA knock down, suggesting that PI3K/Akt pathway mediated the anti-apoptotic effect of PN. Moreover, glutamate receptor stimulation, in conditions known to elicit PN formation (32, 39), caused Akt-Ser⁴⁷³ phosphorylation; conversely, shRNA-mediated Akt knockdown promoted apoptotic death. Together, these results strongly suggest that PN can act as a signaling molecule capable of activating the anti-apoptotic PI3K/Akt pathway in neurons.

In principle, our results might be similar to those obtained by other laboratories, which reported that PN promoted Akt-Ser⁴⁷³ phosphorylation in human skin fibroblasts (Klotz *et al.* 2000). However, the latter study did not contemplate the potential cell death/survival relevance of these findings, and the mechanism suggested for Akt-Ser⁴⁷³ phosphorylation is different from the one we found. Thus, we demonstrate that PN promoted Akt-Ser⁴⁷³ phosphorylation without altering the phosphorylation status of TrkB, an endogenous anti-apoptotic tyrosine kinase receptor for cortical neurons (Hetman *et al.* 1999). In other cellular systems, it has been reported that PN oxidized protein-tyrosine-phosphatases (PTP) leading to the inhibition of receptor tyrosine kinase dephosphorylation (Mallozzi *et al.* 1997; Takakura *et al.* 1999; Klotz *et al.* 2002). In addition, it has been suggested that pre-treatment of human skin fibroblasts with PN impairs subsequent PDGF-induced phosphorylation of its cognate tyrosine kinase receptor (Klotz *et al.* 2000). In contrast, we show that PN and the endogenous TrkB ligand, BDNF, enhance, in an additive fashion, Akt-Ser⁴⁷³ phosphorylation. This is consistent with the notion that PN and BDNF act on different pathways converging in PI3K/Akt activation, in cortical neurons.

Here we show that both, PN-mediated Akt-Ser⁴⁷³ phosphorylation and anti-apoptotic effects were due to its oxidizing potential, and that these phenomena were closely associated with PTEN oxidation. This raises the possibility that our findings could also be attributable to other oxidizing species formed during PN degradation (e.g. hydroxyl radical), or formation from SIN-1 or glutamate (e.g. superoxide). Besides this, over-expression of the wild type PTEN in primary neurons significantly increased apoptotic death when compared with the expression of the inactive, Cys¹²⁴ → Ser¹²⁴ mutated form, confirming the importance

of Cys¹²⁴ residue for PTEN full activity (Lee *et al.* 2002; Kwon *et al.* 2004). Moreover, we show that incubation of wild type PTEN-transfected neurons with PN prevented the excess PTEN-induced apoptotic death. Together, these results indicate that PN – and/or any of its related oxidizing species – oxidizes PTEN, possibly at Cys¹²⁴, hence impairing PTEN to fold properly to acquire its full activity (Lee *et al.* 2002). Interestingly, inhibition of the pro-apoptotic function of PTEN by genetic mutations is known to increase proliferation and the probability of tumorigenesis in certain cycling cells (Lee *et al.* 2002; Cully *et al.* 2006). However, the neurons used in this study are post-mitotic (Almeida *et al.* 2005), making it unlikely that PTEN inhibition by PN could cause increased proliferation. Rather, the anti-apoptotic effect caused by PN-mediated inhibition of PTEN would likely be associated with neuroprotection.

The notion that PN would exert neuroprotection is not in disagreement with the results of other groups (Keynes *et al.* 2004) that have reported the high resistance of neurons against glutamate. We provide a mechanistic explanation for this phenomenon based on the activation of the PI3K/Akt pathway, since Akt knockdown enhanced neuronal vulnerability to glutamate. We are aware that the neuroprotective role that we herein propose for PN argues against the widely held notion that it would be the neurotoxic NO-derivative (Dawson *et al.* 1991). Relatively high concentrations of PN acutely applied to cell cultures and mitochondrial preparations cause oxidation and/or nitration of the complexes of the mitochondrial respiratory chain (Bolaños *et al.* 1995), presumably leading to a loss of function and neurodegeneration. Moreover, it has been recently reported that PN (0.5 mmol/L)-induced apoptosis of PC12 cells was associated with dephosphorylation – leading to inactivation – of Akt (Shacka *et al.* 2006), a result that might be in contradiction with our work. However, it should be mentioned that Akt phosphorylation was assessed, in such study, 16 h after PN additions (Shacka *et al.* 2006). In contrast, we examined Akt phosphorylation 5 min after PN exposure, and neuronal viability within 1 h, a fact that might explain the discrepancy. On the other hand, 3-nitrotyrosine immunostaining has been found in human post-mortem brain sections of patients that suffered from neurodegenerative diseases, such as Alzheimer's disease, suggesting PN formation (Bolaños *et al.* 1997; Sultana *et al.* 2006). However, it has been questioned whether oxidative –and nitrosative– stress is cause, or consequence, of neurodegeneration (Andersen 2004). In view of these considerations and, in light of our findings, it is tempting to suggest that PN formation would represent an early anti-apoptotic cell response aimed at avoiding the propagation of neurodegeneration. Furthermore, our results support the recently highlighted notion (Zhu *et al.* 2006) that PTEN may be a molecular target for interfering with neurodegenerative diseases.

Acknowledgements

J.P.B. is funded by the Spanish Ministry of Science and Education (grant SAF2004-2038) and the Junta de Castilla-León (grant SA081/04). A.A.P. is funded by the Spanish Ministry of Health (Fondo de Investigación Sanitaria, grant FIS06/0794) and the Junta de Castilla-León (grant SA082A05). M.-D.-E. is funded by a Grant from the Universidad de Salamanca (ref. USAL2005-20). D.M-Z is supported by the Spanish Ministry of Health (ISCIII, FIS Grant, ref. PI041418). L.A.-M. was recipient of a fellowship from the Junta de Castilla y León. The authors wish to thank Ms Mónica Resch and Ms Carmen Muñoz for their excellent technical assistance; Prof. R. Pulido (Centro de Investigación Príncipe Felipe, Valencia, Spain) for generously providing wild type and mutated PTEN cDNAs and anti-PTEN (421B) antibody; and Dr Ana Carrera (Centro Nacional de Biotecnología/CSIC, Madrid, Spain) for technical advice.

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