Peroxynitrite activates the phosphoinositide 3-kinase/Akt pathway in human skin primary fibroblasts

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Peroxynitrite is a potent oxidizing and nitrating species formed in a diffusion-limited reaction between nitrogen monoxide and superoxide. It induces apoptosis through unknown mechanisms and is believed to interfere with receptor tyrosine kinase signalling through nitration of tyrosine residues. One pathway emanating from receptor tyrosine kinases is that leading to activation of the anti-apoptotic kinase Akt. In the present study we provide evidence that peroxynitrite, administered to cells using two different delivery systems, results in the dose- and time-dependent activation of Akt. Akt activation is rapid and followed by phosphorylation of glycogen synthase kinase-3, an established substrate of Akt. Akt activation is inhibited in the presence of the phosphoinositide 3-kinase (PI-3K) inhibitors wortmannin and LY294002, and by treatment with the platelet-derived growth factor (PDGF) receptor (PDGFR) inhibitor AG1295, indicating a requirement for PDGFR and PI-3K in mediating peroxynitrite-induced Akt activation. Accordingly, the PDGFR-A and PDGFR-B isoforms were shown to undergo rapid tyrosine phosphorylation on treatment with peroxynitrite. Prior exposure of cells to peroxynitrite interferes with PDGF-induced Akt phosphorylation. Our findings suggest that Akt activation occurs as an acute response to peroxynitrite treatment and could play an important role in influencing cell survival and/or alter the cellular response to other growth regulatory signals.

Key words: oxidative stress, signal transduction, protein kinase B.

INTRODUCTION

Peroxynitrite is the product of the near diffusion-limited reaction between superoxide and nitrogen monoxide ('nitric oxide') [1,2], and is believed to be at least partially responsible for the toxic and detrimental effects of nitric oxide in biological systems [3]. Herein, the term 'peroxynitrite' refers to both the peroxynitrite anion [oxoperoxonitrate(−1)] and peroxynitrous acid [hydrogen oxoperoxonitrate(−1)], which are present at almost equal abundance at physiological pH; the pK_a of peroxynitrous acid is 6.8. It has been estimated that steady-state concentrations of peroxynitrite may be significant in the vicinity of activated macrophages that generate superoxide and nitric oxide concomitantly [4]. Immediate biological effects of peroxynitrite include DNA damage, protein oxidation, lipid peroxidation and nitration, and nitrosation of biological molecules, such as purines or the amino acids tryptophan and tyrosine [5,6]. Cells may react in a variety of ways to peroxynitrite stress, including activation of the c-Jun N-terminal kinase (JNK) and p38 signalling pathways [7–9], the induced expression of stress genes, such as those encoding c-Fos [10], Mn-superoxide dismutase (SOD) [11] and haem oxygenase-1 [12], and the induction of apoptosis [13–15].

Not long after it was first shown that peroxynitrite leads to the nitration of tyrosine residues and the formation of 3-nitrotyrosine *in vivo* [16], it was demonstrated that it affects phosphotyrosine-dependent signalling in cell-free systems [17] and in cell culture [15,18]. It was later shown, however, that nitrated and phosphorylated tyrosines do not necessarily have to be identical, implying that peroxynitrite treatment can not only prevent phosphorylation of tyrosines but also induce it, as demonstrated for src tyrosine kinases in erythrocytes and synaptosomes [19]. In A431 epidermoid carcinoma cells, epidermal growth factor (EGF) receptors (EGFRs) were shown to be a target of peroxynitrite, and covalently dimerized on treatment, with consequences on downstream signalling, as exemplified by phosphorylation of PLC-γ1 [20].

One of the pathways emanating from growth factor receptor activation is the phosphoinositide 3-kinase (PI-3K)/Akt pathway. The proto-oncogene product c-Akt, also termed protein kinase B, has been implicated in anti-apoptotic and proliferative responses to growth factors, such as platelet-derived growth factor (PDGF) and EGF. It can also be activated in response to stressful stimuli, such as heat shock or hydrogen peroxide [21]. PI-3K has been shown to be upstream of Akt, and regulates Akt activity by generating 3′-phosphorylated phosphoinositides that reside in the cell membrane and lead to binding of the pleckstrin homology domain of Akt, concomitant with translocation of Akt to the cell membrane. This relocalization of Akt renders it accessible to regulating kinases, such as 3′-phosphoinositide-dependent kinase-1 (PDK-1) and a PDK-1/PRK-2 complex (where PRK-2 corresponds to protein kinase C-related kinase-2) [22,23] that phosphorylate Akt on Thr^{308} and Ser^{473} [24].

Akt has been shown to be activated by oxidants, such as hydrogen peroxide [25,26] and certain nitric oxide donors [27], but little is known concerning the upstream events leading to Akt activation in these circumstances. The present study was under-

Abbreviations used: CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DCDHF, 2′,7′-dichlorodihydrofluorescein; EBSS, Earle's balanced salt solution; EGF, epidermal growth factor; EGFR, EGF receptor; GSK3, glycogen synthase kinase 3; JNK, c-Jun N-terminal kinase; LY, LY294002; MAP, mitogen-activated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PDK-1, 3′-phosphoinositide-dependent kinase-1; PI-3K, phosphoinositide 3-kinase; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; TBST, Tris-buffered saline containing 0.1% (v/v) Tween-20.

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taken to specifically determine the influence of peroxynitrite and 3-morpholinosydnonimine (SIN-1) on Akt activity. We show here that peroxynitrite, either added to the cells as a bolus of authentic peroxynitrite or generated *in situ* from SIN-1, leads to activation of Akt in human skin primary fibroblasts. We further demonstrate that this activation emanates mostly from PDGF receptors (PDGFRs) that are tyrosine-phosphorylated on treatment with peroxynitrite and that it is mediated by PI-3K. As a consequence, downstream targets of Akt, such as glycogen synthase kinase 3 (GSK3), are phosphorylated. Prior treatment with peroxynitrite leads to inhibition of PDGF- or serum-induced Akt activation. This has important mechanistic implications that are discussed in the present paper.

MATERIALS AND METHODS

Reagents

Cu, Zn-SOD from bovine erythrocytes and catalase from bovine liver were purchased from Sigma (St. Louis, MO, U.S.A.). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), LY294002 (LY) and wortmannin were obtained from Alexis (San Diego, CA, U.S.A.), and AG1295 and AG1478 were purchased from Calbiochem (San Diego, CA, U.S.A.). PDGF-A/B was purchased from R&D Systems (Minneapolis, MN, U.S.A.).

Cell culture and viability measurements

Human primary fibroblasts from neonatal skin were obtained from Clonetics (Walkersville, MD, U.S.A.) and kept in Dulbecco's Modified Eagle's Medium (Gibco BRL, Rockville, MD, U.S.A.), containing 10 % (v/v) fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were used between passages 5 and 17, and grown to 100 % confluence before treatment.

Cell viability was measured using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma) to the corresponding blue formazan. Skin fibroblasts were treated in 24-well plates and incubated with 200 μl/well of MTT (1 mg/ml) in medium for 1–2 h. The reaction was stopped by addition of 200 μl of 10 % (w/v) SDS/0.01 M HCl per well, and the formazan released from the cells by incubation at 37 °C overnight was measured. Absorbance of the supernatant was measured at 570 nm against a background at 700 nm.

Treatment with peroxynitrite and SIN-1

Peroxynitrite was synthesized from sodium nitrite and acidic hydrogen peroxide as described by Koppenol et al. [28]. Briefly, equal volumes of ice-cold 0.6 M $NaNO_2$ and 1.2 M H_2O_2/0.6 M HCl were combined and instantly added to the same volume of cold 1.5 M NaOH. Residual hydrogen peroxide was removed by passage over MnO_2. After freeze fractionation, a 240 mM solution of peroxynitrite was obtained. Concentrations were determined spectrophotometrically at 302 nm ($\epsilon = 1670\ M^{-1} \cdot cm^{-1}$). For treatments with peroxynitrite, skin fibroblasts were washed once and then covered with PSG buffer (100 mM potassium phosphate, 10 mM NaCl and 5 mM glucose, pH 7.4) at ambient temperature. Peroxynitrite [prepared from a stock in 0.1 % (w/v) NaOH] was added as a bolus, followed instantly by tilting and shaking of the culture dish. To avoid increases in pH, peroxynitrite was added in volumes that were a maximum of 1 % of the culture volume to yield the given final concentrations. Following incubation for 5 min serum-free medium was added to the cells, which were kept in an incubator for the desired time.

SIN-1 (Sigma) was dissolved and diluted in Earle's balanced salt solution (EBSS; Gibco BRL), and cells were incubated at 37 °C for the desired time. Incubations were with either 1 ml/30 mm dish or 2 ml/60 mm dish.

Peroxynitrite generation during incubation of 5 mM SIN-1 in EBSS at 37 °C was nearly linear for 1 h, and was estimated to be 70 nmol/min per ml during that time. Generation of peroxynitrite from SIN-1 was determined by spectrophotometrically following the rate of oxidation of 2′,7′-dichlorodihydrofluorescein (DCDHF) to 2′,7′-dichlorofluorescein [29], and relating these data to a standard curve of DCDHF oxidation by authentic peroxynitrite in EBSS at 37 °C. A stock solution of DCDHF was prepared by hydrolysing DCDHF diacetate (Sigma) in 0.2 M NaOH.

In experiments with inhibitor treatments, the cells were pre-incubated for 30–75 min with either DMSO (control) or the appropriate inhibitor, which were also present during treatment with SIN-1 or peroxynitrite and during post-incubation. To control for interactions between SIN-1, its decomposition products or peroxynitrite and the employed inhibitors, cell lysates were routinely analysed for total protein nitration. The lysates were also checked for phosphorylation of p38, which we found to be induced by addition of SIN-1 or peroxynitrite to human skin fibroblasts and which is independent of the targets of the inhibitors used, i.e. PDGFR and PI-3K.

Western-blot analysis

Western blotting was performed as described previously [30]. For detection of phosphorylated (Ser^{473} or Thr^{308}) and total Akt or GSK3-α/β, rabbit polyclonal antibodies from New England Biolabs (Beverly, MA, U.S.A.) were used at the dilutions recommended by the supplier. Phosphorylated extracellular signal-regulated protein kinase/mitogen-activated protein (MAP) kinases were detected with the polyclonal anti-(active-MAP kinase) antibody from Promega (Madison, WI, U.S.A.), and 3-nitrotyrosine was detected with a monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.). All incubations were carried out in 5 % (w/v) dry milk in Tris-buffered saline containing 0.1 % (v/v) Tween-20 (TBST).

Immunoprecipitations

Cells were treated in 60 mm dishes. After treatment, cells were washed once and lysed in 250 μl of lysis buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % (v/v) Triton, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM PMSF and 1 μg/ml leupeptin] per dish by incubating on ice for up to 1 h. Lysed cells were collected with a cell lifter and transferred to Eppendorf tubes. The crude lysates of at least three dishes were combined for one sample. Lysates were frozen at −80 °C, thawed and centrifuged to pellet non-soluble fractions. The resulting supernatants were transferred to fresh Eppendorf tubes, and protein concentration was determined using the detergent-compatible Bio-Rad Protein assay.

EGFR was immunoprecipitated from 100–200 μg of total protein in a total volume of 200 μl of lysis buffer by the addition of 0.7–1 μg of rabbit polyclonal anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) per 100 μg of total protein. After incubation on ice for up to 2 h, Protein

A–Sepharose ('4 Fast Flow'; Pharmacia, Uppsala, Sweden) that had been pre-equilibrated in lysis buffer was added, and the suspension was rotated end-over-end at 4 °C overnight. The samples were centrifuged at 20000 ***g*** at 4 °C for 20 s and washed twice with lysis buffer and once with PBS. After addition of SDS sample buffer to the washed Protein A pellets, samples were boiled and applied to an 8 % (w/v) polyacrylamide gel, buffered in Tris/glycine, followed by blotting on to PVDF membranes. Detection of phosphorylated tyrosine residues was with monoclonal anti-phosphotyrosine antibody (4G10; Upstate Biotechnology). Membranes were blocked for 1 h in 5 % (w/v) BSA in TBST, briefly washed with TBST and incubated with the primary antibody diluted 1:1000 in 1 % BSA in TBST at 4 °C overnight. The secondary antibody was diluted in TBST. After detection, membranes were stripped and re-probed with the anti-EGFR antibody.

Immunoprecipitations of PDGFR-A and PDGFR-B were performed similarly, but using antibodies against PDGFR-A (Upstate Biotechnology) and PDGFR-B (Santa Cruz Biotechnology) and an anti-(PDGFR-A/B) antibody (Upstate Biotechnology) for reprobing of the membrane.

RESULTS

Activation of Akt by peroxynitrite

Treatment of confluent human skin primary fibroblasts with SIN-1 in EBSS for 1 h lead to activation of Akt, as shown by the phosphorylation of Ser^{473}, which is essential for activation (Figure 1A). Thr^{308}, a second residue critical for full activation of Akt, was also phosphorylated upon addition of SIN-1 (see Figure 3A). This activation was visible at approx. 30 min after treatment with SIN-1 and persisted for at least 1 h after removal and replacement of the drug with serum-free medium. Figure 1(A) shows phosphorylation of Akt after a 60 min incubation with SIN-1 and 30 min after removal of SIN-1. Since SIN-1 only indirectly generates peroxynitrite through a sequential release of superoxide and nitric oxide and their diffusion-limited reaction, we sought to verify that peroxynitrite, rather than the SIN-1 decomposition product, SIN-1C, or superoxide or nitric oxide were responsible for this activation. Thus the nitric oxide scavenger CPTIO (300 μM), and Cu,Zn-SOD (100 units/ml) in combination with catalase (500 units/ml) were tested for their effects. Either addition not only diminished nitration of protein tyrosines in the treated cells but also completely abolished Akt phosphorylation. Hence, both nitric oxide and superoxide are essential for the observed activation, indicating that it is highly likely that peroxynitrite, the reaction product of both, is the active agent leading to Akt activation.

To further confirm this, authentic peroxynitrite was added to confluent cells, and 15 or 30 min after treatment cells were lysed and analysed for phosphorylation of Akt (Figure 1B). An increase in Akt phosphorylation was apparent with 100 μM peroxynitrite, with maximum activation occurring at a concentration of 500 μM.

Treatment of cells with 3 and 5 mM SIN-1 for 1 h resulted in a significant reduction in cell viability (results not shown). Cell viability was reduced by 45 % and 70 % respectively (results not shown) 24 h after treatment. As with Akt activation and tyrosine nitration, toxicity was completely abolished in the presence of either CPTIO or SOD/catalase. The concentrations of peroxynitrite used were only mildly toxic to the cells, with only a 25 % loss of viability 24 h after treatment at 500 μM (results not shown).

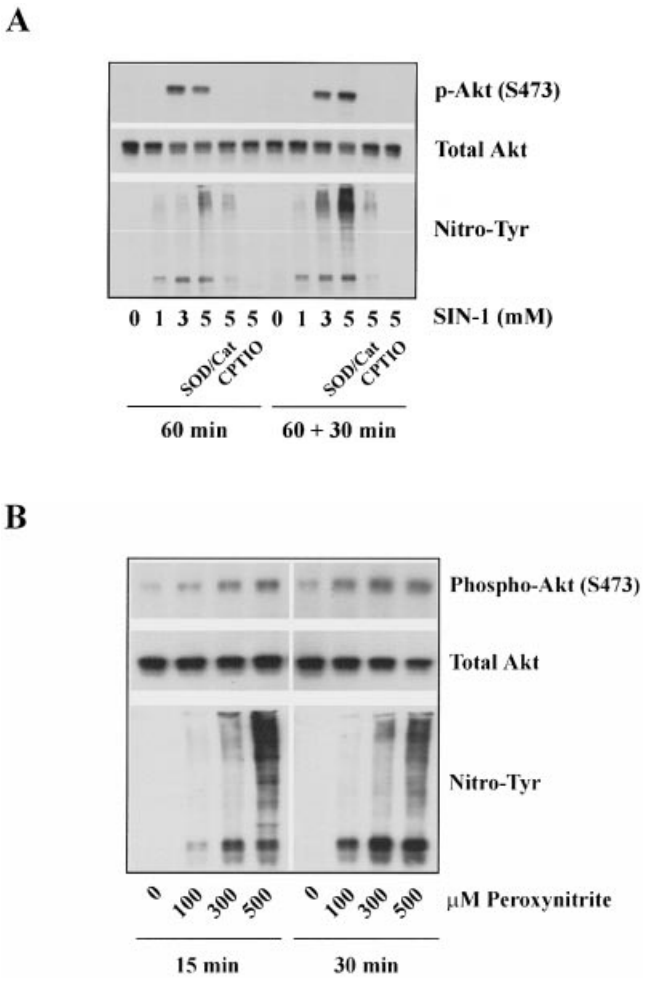

Figure 1 Phosphorylation of Akt induced by SIN-1 and peroxynitrite

(**A**) Human skin fibroblasts were incubated with various concentrations of SIN-1 in the presence or absence of SOD (100 units/ml) plus catalase (Cat; 500 units/ml), or the nitric oxide scavenger CPTIO (300 μM), for 1 h and either lysed and processed for Western-blot analysis directly (left-hand side of Figure) or incubated in serum-free medium for another 30 min (right-hand side of Figure) before lysis. The Figure shows phosphorylated Akt (p-Akt) and total Akt, as well as total protein-tyrosine nitration (Nitro-Tyr), as detected by Western-blot analysis using appropriate antibodies (see the Materials and methods section). (**B**) Fibroblasts were treated with a single bolus of various doses of authentic peroxynitrite to yield the given final concentrations. Again phosphorylated Akt (Phospho-Akt) and total Akt, as well as total protein-tyrosine nitration, were detected by Western-blot analysis. A peroxynitrite concentration of 0 μM corresponds to pre-decomposed peroxynitrite (500 μM). This control was used to check for an effect of any peroxynitrite decomposition products.

Phosphorylation of GSK3

One of the substrates of Akt *in vivo* and *in vitro* is GSK3. Phosphorylation by Akt inhibits the enzyme and its action on downstream targets such as glycogen synthase, c-Jun and β-catenin (for review, see [31]). To further verify the functional significance of Akt phosphorylation in cells exposed to peroxynitrite we examined whether GSK3 also became phosphorylated following treatment. Using an antibody specific for the phosphorylated forms of GSK3, we observed an increase in the amounts of phosphorylated GSK3-α and GSK3-β after treatment with SIN-1, which was blocked by the presence of CPTIO or SOD/catalase (Figure 2A). Enhanced GSK3 phosphorylation

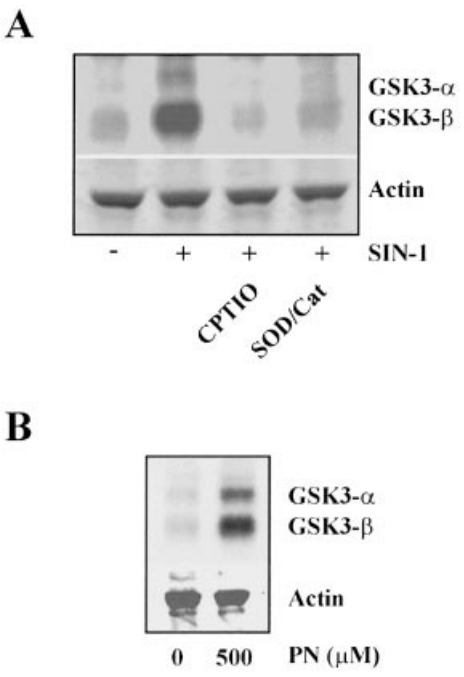

Figure 2 Phosphorylation of GSK3-α and GSK3-β by SIN-1 and peroxynitrite

Human skin fibroblasts were treated with 5 mM SIN-1 (**A**) or peroxynitrite (PN; **B**) as described in the legend to Figure 1, lysed and analysed by Western blotting for detection of phosphorylated isoforms of GSK3. Actin expression corresponds to the prominent 42 kDa band in gels stained with Coomassie Brilliant Blue, and verifies that equivalent amounts of protein were present in the different lanes.

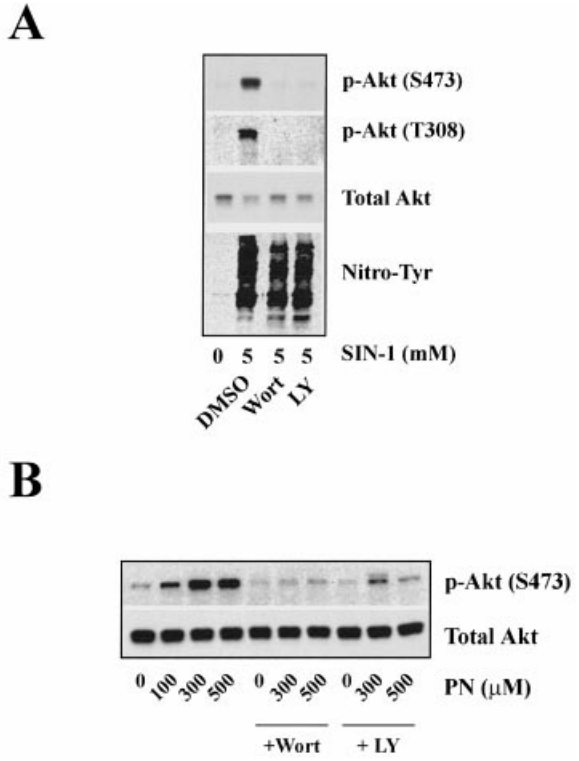

Figure 3 Effect of PI-3K inhibitors on Akt phosphorylation by SIN-1 and peroxynitrite

Wortmannin (Wort) was used at a concentration of 200 nM and LY was used at a concentration of 20 μM. Western blots are shown with immunodetection of two phosphorylated forms of Akt, total Akt and total protein-tyrosine nitration. (**A**) Results obtained with SIN-1 treatment. (**B**) Results obtained with authentic peroxynitrite treatment.

was also evident following addition of authentic peroxynitrite (Figure 2B).

Role of PI-3K and PDGFRs

As pointed out above, activation of Akt most often occurs via a PI-3K-dependent mechanism. However, Akt can be phosphorylated independently of PI-3K, such as through the protein kinase A pathway [32] or as as result of increases in cytosolic Ca^{2+} levels [33]. To test the role of PI-3K in Akt activation by peroxynitrite, we treated cells with selective inhibitors of PI-3K, wortmannin (200 nM) and LY (20 μM), before adding SIN-1 or authentic peroxynitrite. Phosphorylation of Akt was completely abolished by pretreatment with the PI-3K inhibitors (Figure 3). The inhibition of phosphorylation was not due to an interaction between the respective inhibitor and either SIN-1 or peroxynitrite, as tyrosine nitration of proteins, a marker for the action of peroxynitrite, was not affected. In addition, neither of the PI-3K inhibitors interfered with peroxynitrite-induced activation of p38, which occurs independently of PI-3K (results not shown).

Next, we investigated the involvement of growth factor receptor tyrosine kinases (as upstream activators of PI-3K) in phosphorylation of Akt by SIN-1 and peroxynitrite. First we examined whether PDGFRs and EGFRs undergo tyrosine phosphorylation in response to SIN-1 or peroxynitrite. As human skin fibroblasts express both PDGFR-A and PDGFR-B [34], both forms of the receptor were immunoprecipitated and examined for tyrosine phosphorylation by Western blotting. As shown in Figures 4(A) and 4(B), both PDGFR-A and PDGFR-B were tyrosine-phosphorylated after addition of SIN-1. This phosphorylation was visible at 15 min after addition of SIN-1, reached a maximum at 30–45 min, and persisted for at least 60 min. Phosphorylation was completely inhibited in the presence of either the nitric oxide scavenger CPTIO or SOD/catalase (Figures 4A and 4B), pointing to the formation of peroxynitrite from the nitric oxide and superoxide released from SIN-1 as a prerequisite for receptor phosphorylation. To ensure equal loading, blots were stripped after detection of tyrosine phosphorylation and re-probed with an antibody capable of recognizing both PDGFR isoforms.

To examine the possible link between autophosphorylation of the PDGFR and phosphorylation of Akt, cells were pretreated with the PDGFR kinase inhibitor AG1295 before addition of SIN-1 and peroxynitrite. As shown in Figure 5, AG1295 strongly inhibited phosphorylation of Akt induced by addition of SIN-1 (Figure 5A), as well as that induced by addition of authentic peroxynitrite (Figure 5B).

Another reactive species, hydrogen peroxide, has been shown to lead to activation of Akt via EGFR activation [26]. For this reason, we examined whether EGFR also underwent phosphorylation following addition of SIN-1. Indeed, EGFR was phosphorylated by addition of SIN-1 to human skin fibroblasts (Figure 6A), the time-course of phosphorylation being identical with that found for PDGFR-A and PDGFR-B. Furthermore, as with the PDGFRs, addition of CPTIO or SOD/catalase completely abolished this effect.

To determine if EGFR phosphorylation was required for phosphorylation of Akt by SIN-1, cells were pretreated with the

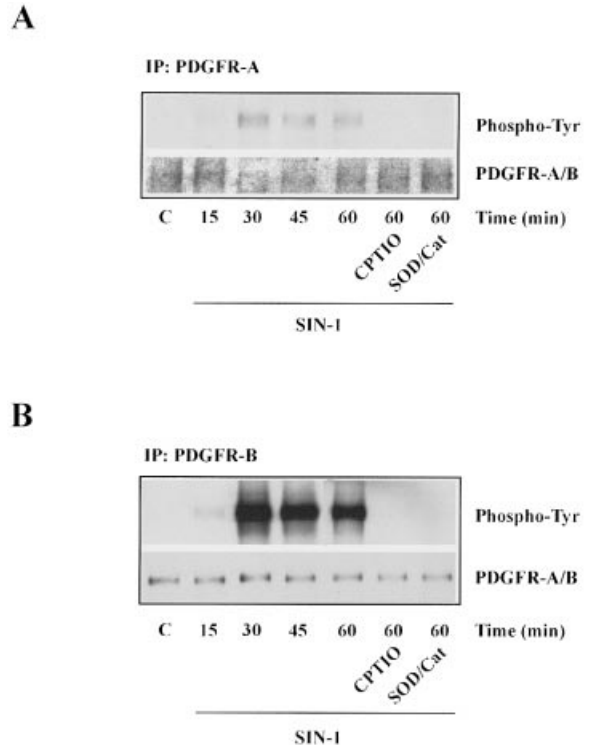

Figure 4 Tyrosine phosphorylation of PDGFRs following SIN-1 treatment

Cells were treated with 5 mM SIN-1 for the given times, after which PDGFR-A (**A**) and PDGFR-B (**B**) were immunoprecipitated (IP) from cell lysates and the precipitates separated by SDS/PAGE, and blotted on to PVDF membranes. The membranes were probed with an antibody specific for phosphotyrosine residues. For loading controls, the blots were stripped and re-probed with an antibody recognizing both PDGFR isoforms. C, control.

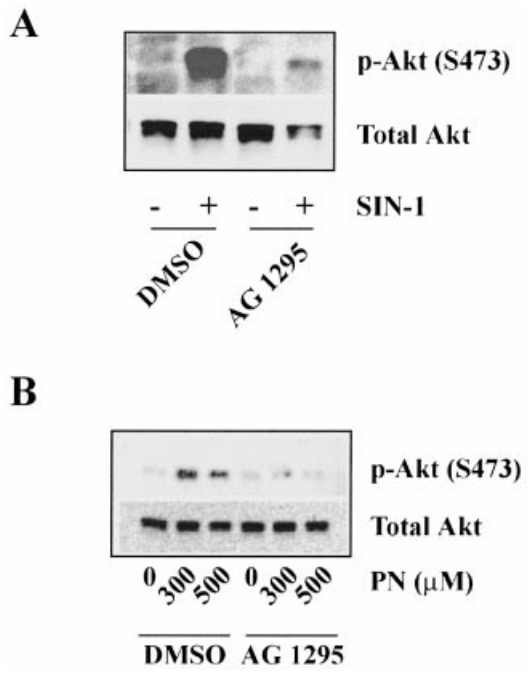

Figure 5 Activation of Akt by SIN-1 (A) and peroxynitrite (B) is mediated by PDGFR tyrosine kinase

Cells were treated with 3 mM SIN-1 (**A**) or the given concentrations of peroxynitrite (PN; **B**) in the absence or presence of the PDGFR inhibitor AG1295 (10 μM). DMSO served as a vehicle control. Akt activation was assessed by Western-blot analysis detecting phosphorylation of Ser^{473}.

EGFR inhibitor AG1478. Though capable of completely blocking Akt activation induced by EGF, AG1478 had only a minor effect on SIN-1-induced activation (Figure 6B). It should be mentioned that we saw some inhibition in some experiments; yet there was no such reproducibility as with the PDGFR inhibitor. Hence, the cascade leading to the activation of Akt by SIN-1 is apparently mainly initiated at the PDGFR level.

Peroxynitrite-induced Akt activation interferes with subsequent Akt activation by growth factors

As peroxynitrite is a strong oxidizing and nitrating species, it cannot be excluded that treatment of cells leads to modification of receptors and other signalling molecules. One would assume that, as a consequence, the pathway leading to phosphorylation of Akt will either be more readily stimulated due to inactivation of regulating enzymes, such as phosphatases, or will be modified in such a way that regular signalling along this pathway is no longer possible.

To test for these possible scenarios, we treated human dermal fibroblasts with SIN-1 or peroxynitrite, followed by the addition of PDGF or fetal calf serum. As shown in Figure 7, treatment of cells with either PDGF (Figure 7A) or serum (Figure 7B) resulted in strong phosphorylation of Akt that was attenuated by pretreatment with either SIN-1 (1–5 mM) for 1 h or peroxynitrite (up to 500 μM). SIN-1 was effective at concentrations of 3 and 5 mM, and the inhibition of PDGF- or serum-induced Akt phosphorylation was reversed by the addition of CPTIO or

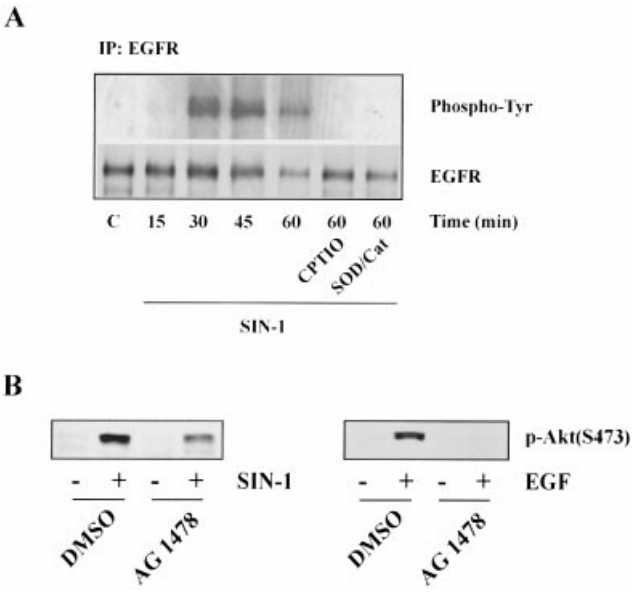

Figure 6 Role of EGFR in SIN-1-induced Akt phosphorylation

(**A**) Cells were treated with 5 mM SIN-1 for 1 h, after which EGFR was immunoprecipitated from the lysates, and examined by Western blotting using an anti-phosphotyrosine antibody. The stripped blot was re-probed with an anti-EGFR antibody to verify equal amounts of proteins in the different lanes. (**B**) Cells were treated with 3 mM SIN-1 (left panel) or 200 ng/ml EGF (right panel) in the absence or presence of the EGFR inhibitor AG1478 (10 μM). Western-blot analysis was used to detect phosphorylated Akt.

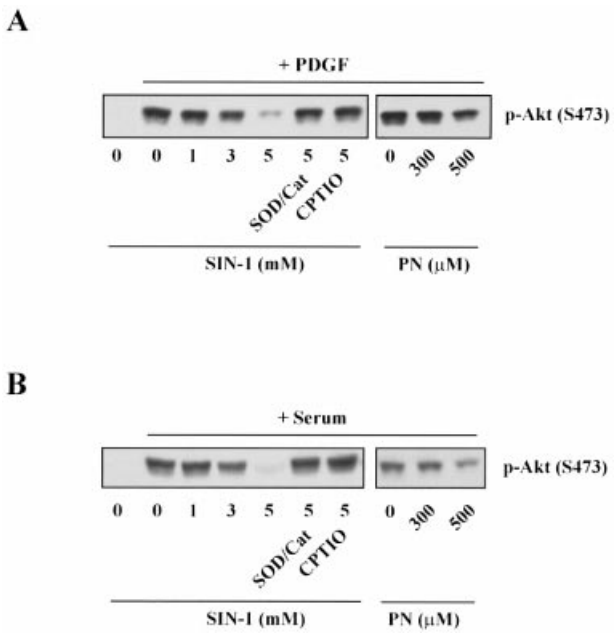

Figure 7 Effect of SIN-1 and peroxynitrite on (A) PDGF- and (B) serum-induced phosphorylation of Akt

Skin fibroblasts were treated with various concentrations of SIN-1 for 1 h or with boluses of peroxynitrite, and were then treated with PDGF-A/B (100 ng/ml) or fetal calf serum for 30 min before lysis and processing for Western blotting. The first lane is the no-treatment control. CPTIO and SOD/catalase were added as in Figure 1(A).

SOD/catalase. Addition of authentic peroxynitrite at 500 μM had a similar effect on activation of Akt by PDGF or serum, although its inhibitory effect was not as great as that seen with SIN-1. This is due perhaps to the fact that authentic peroxynitrite is added to the cells as a one-time bolus, whereas peroxynitrite is continuously generated when employing SIN-1.

DISCUSSION

Peroxynitrite has been shown to induce apoptosis in a variety of cell systems [13–15]. Consistent with this effect, several kinases whose activation is often associated with apoptosis, including the stress-activated kinases p38 [7,8] and JNK [7,9], are activated on exposure to peroxynitrite. In the present study we have demonstrated that peroxynitrite also activates a pathway leading to the activation of Akt, a kinase that is at least in part responsible for the anti-apoptotic effects of growth factors and other proliferative stimuli. The PI-3K/Akt pathway has also been shown to be activated by other oxidants, including hydrogen peroxide [25,26] and nitric oxide [27], but this appears to be a unique feature of oxidant injury, as a variety of other stresses, including ceramide, hyperosmotic stress, and ionizing radiation [35], are associated with a reduction in Akt activity.

Peroxynitrite is known to nitrate protein-tyrosine residues, and this nitration impairs phosphorylation of tyrosines crucial for phosphotyrosine signalling [15–19]. However, studies using epidermoid carcinoma cells indicated that while exposure to peroxynitrite leads to nitration of EGFR tyrosine residues, it does not inhibit receptor autophosphorylation [20]. The present work has shown that exposure of human skin primary fibroblasts to peroxynitrite, derived from SIN-1 hydrolysis, leads to the phosphorylation of not only the EGFR but also the two expressed types of PDGFR (Figures 4 and 6A). Hence, there is clear induction rather than repression of tyrosine phosphorylation by peroxynitrite in our system.

In a previous study examining Akt activation in response to oxidative insults [26], we found that the EGFR plays a primary role in mediating Akt activation by hydrogen peroxide, as activation was completely blocked in the presence of specific inhibitors of EGFR phosphorylation. In the present study, we show that the EGFR also undergoes phosphorylation in response to peroxynitrite, but this appears not to be important for Akt activation by peroxynitrite, as the same inhibitors of EGFR phosphorylation did not alter the response to peroxynitrite. Instead, AG1295, a specific inhibitor of PDGFR phosphorylation, was found to abolish Akt activation, indicating an important role for this growth factor receptor in mediating the response. It should be emphasized that our prior studies with hydrogen peroxide employed HeLa cells, whereas the current studies were performed with human skin fibroblasts. Thus whether the differences in growth factor signalling pathways (EGFR versus PDGFR) employed to mediate hydrogen peroxide- and peroxynitrite-induced Akt activation reflect true agent selectivity (hydrogen peroxide versus peroxynitrite) or differences in the cell types used remains to be determined. In any event, our current studies taken together with our previous findings indicate an important role for growth factor receptor tyrosine kinases in transducing signals initiated by oxidative insults at the cell membrane to downstream events leading to Akt activation.

The mechanisms contributing to PDGFR activation following exposure to peroxynitrite remain to be resolved. One possible way is through inhibition of a regulating phosphatase. All protein tyrosine phosphatases known so far rely on the presence of a cysteine residue in their active site [36]. Oxidation of this cysteine residue inactivates the phosphatase, leading to a net increase in phosphorylation of proteins normally controlled by the phosphatase. Various oxidants have been shown to activate growth factor signalling pathways and associated downstream events, at least in part, via such a mechanism [37,38]. In addition, peroxynitrite can inactivate a variety of phosphatases *in vitro* [39]. While inhibition of phosphatase activity could contribute to activation of PI-3K/Akt by peroxynitrite, our finding that prior peroxynitrite treatment renders cells refractory to subsequent Akt activation by growth factors (Figure 7) indicates that this cannot be the sole mechanism responsible for activation of the kinase. If the inhibition of a regulating phosphatase by peroxynitrite were responsible for the increased Akt phosphorylation, then phosphorylation would not be expected to be inhibited by peroxynitrite pretreatment, but rather would be expected to be enhanced due to the loss of negative regulation.

The PI-3K/Akt pathway has anti-apoptotic properties, as has been shown in a variety of systems with overexpression of constitutively active or dominant negative mutants of Akt (for discussion and references therein see [21]). This activity is believed to be due to the *in vivo* substrates of Akt, most of which can be linked to apoptosis. Among these substrates are the Bcl-2 relative Bad, caspase 9, forkhead transcription factors, c-Raf, and GSK3, all of which are inactivated through phosphorylation by Akt [21,40–42]. Furthermore, Akt has been linked to activation of the nuclear factor κB pathway in some cell lines [43,44]. Whether activation of Akt activation in response to peroxynitrite is important in influencing the survival of peroxynitrite-treated cells remains to be evaluated. We have recently reported that activation of Akt in response to hydrogen peroxide treatment significantly enhances cell survival [26], and it is possible that Akt activation serves a similar function in peroxynitrite-treated cells.

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