0026-895X/03/6403-557–569$7.00
MOLECULAR PHARMACOLOGY
Vol. 64, No. 3
2316/1084483
Mol Pharmacol 64:557–569, 2003
Printed in U.S.A.

Critical Role for Akt1 in the Modulation of Apoptotic Phosphatidylserine Exposure and Microglial Activation

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Received December 3, 2002; accepted May 14, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Biological targets for neurodegenerative disease that focus on the intrinsic maintenance of cellular integrity and the extrinsic prevention of phagocytic cellular disposal offer the greatest promise for therapeutic intervention. Protein kinase B (Akt1), a serine-threonine kinase closely involved in cell growth and survival, offers a strong potential to address both intrinsic and extrinsic mechanisms of neuronal injury. We demonstrate that overexpression of a constitutively active form of Akt1 (myristoylated Akt1) in differentiated SH-SY5Y neuronal cells provides intrinsic cellular protection against apoptotic genomic DNA destruction and membrane phosphatidylserine (PS) exposure. Transfection of SH-SY5Y cells with a plasmid encoding a kinase-deficient dominant-negative Akt1 eliminates cytoprotection, suggesting that activation of Akt1 is necessary and sufficient to prevent apoptotic destruction. Apoptotic neuronal membrane PS exposure provides a unique pathway for Akt1 to offer extrinsic cellular protection and block microglial activation, because independent cotreatment with an anti-PS receptor neutralizing antibody could also prevent microglial proliferation. Akt1 maintains nuclear DNA integrity and membrane PS exposure through the specific inhibition of caspase 3-, 8-, and 9-like activities that were linked to mitochondrial membrane potential and cytochrome *c* release. Our work elucidates a novel capacity for Akt1 to maintain cellular integrity through a series of cysteine protease pathways and to uniquely regulate microglial activation through the modulation of membrane PS residue externalization.

Identification of biological targets that can regulate "pro-apoptotic" cellular pathways in the nervous system may offer new therapeutic avenues against neuronal injury. However, therapeutic modulation of potential biological targets must address both intrinsic mechanisms of cellular apoptosis that impact upon neuronal cellular integrity and extrinsic pathways of cellular disposal that can illicit inflammatory activation of microglia with subsequent neuronal phagocytosis (Maiese and Vincent, 2000; Hoffmann et al., 2001). One attractive target that may modulate both intrinsic and extrinsic pathways of injury is protein kinase B, also referred to as PKBα or Akt1. The serine-threonine kinase Akt1 is intimately linked to cell growth and survival. As a downstream target of phosphoinositide 3 kinase (PI 3-K), the cytosolic protein Akt1 translocates to the cell membrane and subsequently becomes activated through phosphorylation by phosphoinositide-dependent kinase 1 (Wick et al., 2000). Once phosphorylated, Akt1 modulates the activity of several substrates that may influence cell survival, such as Bad, IκB kinase α, the forkhead transcription factor, and the glycogen synthase kinase-3β. Increased expression of phosphorylated Akt can occur in a variety of nervous system insults, such as during free radical exposure (Matsuzaki et al., 1999; Chong et al., 2002a), trauma (Murashov et al., 2001), and ischemia (Sakurai et al., 2001). More importantly, enhanced activity of Akt can confer resistance against hypoxic injury (Scott et al., 2002), whereas loss of Akt activity leads to cellular death (Namikawa et al., 2000). Yet the underlying mechanisms that may determine the ability of Akt1 to protect against intrinsic mechanisms of neuronal apoptosis require further definition. Furthermore and of equal significance, the potential ability of Akt1 to confer protection against extrinsic pathways of cellular disposal during microglial activation has not been previously addressed.

This research was supported by grants (all to K.M.) from the American Heart Association (National), Janssen Neuroscience Award, Johnson and Johnson Focused Investigator Award, LEARN Foundation Award, MI Life Sciences Challenge Award, and National Institute of Environmental Health Sciences grant P30-ES06639.

ABBREVIATIONS: PI 3-K, phosphoinositide 3 kinase; NO, nitric oxide; PS, phosphatidylserine; RA, retinoic acid; myr-Akt1, myristoylated (active) form of Akt1; dn-Akt1, dominant-negative Akt1; MEM, Eagle's minimum essential α medium; PCNA, proliferating cell nuclear antigen; BrdU, bromodeoxyuridine; PSR, phosphatidylserine receptor; NOC-9, 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; TUNEL, terminal deoxynucleotidyl transferase nick-end labeling; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; Ac, *N*-acetyl; PNA, *p*-nitrolanilide; Z, *N*-benzyloxycarbonyl; FMK, fluoromethyl ketone; p-Akt1, phosphorylated Akt1; PC, phosphatidylcholine; Ab, antibody.

To promote the development of Akt1 as a protectant against neurodegenerative disease, it is first critical to understand the cellular pathways that may mediate cellular injury and that are subsequently susceptible to modulation by Akt1. In this regard, free radicals, such as nitric oxide (NO), have been established as important pathological components of several neuronal disorders, such as Alzheimer's disease and cerebral ischemia (Maiese and Vincent, 2000; Anderson et al., 2001). Similar to other oxidants, NO can lead to cellular apoptosis through either direct pathways, such as producing single or double-strand breaks in DNA (Martin and Liu, 2002), or secondary pathways, such as endonuclease activation (Vincent and Maiese, 1999b), intracellular acidification (Vincent et al., 1999), mitogen-activated protein kinases (Ghatan et al., 2000), or peroxynitrite (Oka et al., 2000). In addition, NO can trigger the induction of two independent apoptotic pathways that consist of nuclear DNA degradation and the exposure of membrane phosphatidylserine (PS) residues (Maiese and Vincent, 2000; Hoffmann et al., 2001; Lin and Maiese, 2001). Although DNA degradation may immediately alter cellular integrity (Jessel et al., 2002), the exposure of membrane PS residues can lead to acute cellular inflammation (Dombroski et al., 2000) and microglial phagocytosis of viable neurons (Maiese and Vincent, 2000; Hoffmann et al., 2001).

The cytoprotective role of Akt1 may require the modulation of a series of downstream cellular pathways. In particular, cellular release of NO can lead to the activation of a cascade of executioner cysteine proteases (caspases) that play crucial roles during genomic DNA degradation and membrane PS exposure. For example, through a series of cellular events, caspase 9 leads to the activation of caspase 3 (Li et al., 1997), which may require the intermediate activation of caspase 8. Subsequently, caspase 3 can lead to both DNA fragmentation and membrane PS exposure (Takahashi et al., 1999; Lin and Maiese, 2001). The upstream trigger for the initiation of the executioner cysteine proteases may, in part, involve mitochondrial membrane depolarization and the opening of mitochondrial permeability transition pores (Bal-Price and Brown, 2000; Chong et al., 2002b). Cytochrome *c* is then released from mitochondria and leads to caspase activation. Given the potentially unique role for Akt1 to modulate both intrinsic and extrinsic pathways of cellular injury, we investigated the underlying cellular mechanisms controlled by Akt1 that may determine both the maintenance of neuronal cellular integrity and the prevention of phagocytic cell disposal as a basis for the future development of cytoprotective strategies in the nervous system.

Materials and Methods

Human Neuroblastoma SH-SY5Y Cell Culture and Differentiation. Human adrenergic neuroblastoma SH-SY5Y cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in regular Eagle's minimum essential α medium (MEM; Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum, 1 mM pyruvate, 1.5 g/l sodium bicarbonate, 100 IU/ml penicillin, 100 μg/ml streptomycin at 37°C in 95%/5% (v/v) mixture of humidified atmospheric air and CO_2. Cell suspension was prepared at a density of 3 to 4×10^4 (24-well plate) or 1 to 1.5×10^5 (35-mm^2 Petri dish). When 50 to 60% confluent, cells were differentiated by MEM containing 10 μM all-*trans* retinoic acid (RA) (Sigma, St. Louis, MO) for 48 h. RA was dissolved in dimethyl sulfoxide to overcome the low solubility of RA in aqueous media. Experiments were initiated until cells grew to 60 to 70% confluence between passages 4 and 10 after differentiation.

Stable Transfection of myr-Akt1 cDNA Construct into SH-SY5Y Cells. Stable multiple SH-SY5Y clones overexpressing the myristoylated (active) form of Akt1 (myr-Akt1) were generated by transfecting the cells with a cDNA construct under the control of a cytomegalovirus promoter with cDNA (6.89 kilobases) containing sequences corresponding to amino acids 1 to 11 of avian *c-rsc* at the 5′ end and a Myc-His tag at the 3′end of the mouse Akt1 open reading frame (Upstate Biotechnology, Lake Placid, NY) by lipofection with LipofectAMINE Plus reagent (Invitrogen). Subsequent selection of the transfectants was performed with 400 μg/ml Geneticin (Invitrogen) 48 h later. Stable clones were identified, collected, and expanded over a 3- to 4-week course with transfection efficiency equal to approximately 98% ($n = 20$). Individual clones were evaluated independently and characterized by phosphorylated Akt1 expression on Western analysis and by immunocytochemistry detection with Myc Tag (anti-Myc rabbit polyclonal IgG (1:1000); Upstate Biotechnology) conjugated to biotinylated anti-rabbit IgG (1:50) and fluorescein avidin (1:50) (Vector Laboratories, Burlingame, CA).

Transfection of Dominant-Negative Akt1 cDNA Construct into SH-SY5Y Cells. SH-SY5Y cells overexpressing a dominant-negative Akt1 mutant (dn-Akt1) that lacked kinase activity were generated by transfecting the cells with a cDNA construct under the control of a cytomegalovirus promoter with cDNA that contains a substitution of methionine (ATG) for lysine (AAG) at residue 179 in pUSEamp and a Myc-His tag at the 3′-end of the mouse Akt1 open reading frame (K179M mutant; Upstate Biotechnology, Lake Placid, NY) by lipofection with LipofectAMINE reagent (Invitrogen) according to the manufacturer's instruction. Briefly, cells were seeded into 35-mm dishes at a concentration of 1 to 1.5×10^5 and transfected with 1 μg of dn-Akt1 cDNA once the monolayer was 70 to 80% confluent. After 72 h in culture, cells were washed with MEM growth medium supplemented with 10% heat-inactivated fetal bovine serum and differentiated with 10 μM all-*trans*-RA for 48 h before experimentation. Clones were characterized by the absence of phosphorylated Akt1 expression on Western analysis and by immunocytochemistry detection with Myc Tag [anti-Myc rabbit polyclonal IgG (1:1000); Upstate Biotechnology] conjugated to biotinylated anti-rabbit IgG (1:50) and fluorescein avidin (1:50; Vector Laboratories, Burlingame, CA) to yield a transfection efficiency equal to approximately 24% ($n = 20$).

Microglia Cell Cultures, Assessment of Microglial Activation, Proliferation, and the Microglial Phosphatidylserine Receptor. Microglia were obtained from the cerebral cortex of E-19 Sprague-Dawley rat pups (Giulian and Baker, 1986). Briefly, cerebral cortex cells were mechanically dissociated, seeded in 75-cm^2 plastic flasks at a density of 8.5×10^6 cells per flask, and maintained with Dulbecco's modified Eagle's/Ham's F-12 medium (Invitrogen) containing 10% fetal bovine serum (ICN, Aurora, OH). Microglia were purified from mixed cultures with reciprocal shaking at 180 rpm for 15 h and then reseeded at 10^5 cells/ml for cell adhesion of 3 h duration to yield an almost pure preparation of microglia (>98%). Microglial cells were identified by α-naphthyl acetate esterase, OX-42, and isolectin B4 from *Griffonia simplicifolia* (Sigma, St. Louis, MO). The cells did not stain for glial fibrillary acidic protein (Sigma, St. Louis, MO).

Microglia were conditioned for 3 h by media from either wild-type or SH-SY5Y cells overexpressing myr-Akt1 24 h after NO exposure. Proliferating cell nuclear antigen (PCNA) staining for microglial activation (Williams et al., 2002) and bromodeoxyuridine (BrdU) staining for microglial proliferation (Martinez-Contreras et al., 2002) was performed with anti-mouse monoclonal antibody PCNA (1:100) or BrdU (1:100) conjugated with biotinylated anti-mouse IgG (1:50) and visualized through fluorescein avidin (1:50) for PCNA and Texas Red streptavidin (Vector Laboratories) for BrdU. BrdU (10 μM) and fluorodeoxyuridine (1 μM; Sigma, St. Louis, MO) were applied 1 h

before the time of fixation. For detection of microglial PSR (Hoffmann et al., 2001), microglia were incubated for 15 h with neuronal media and then exposed to mouse anti-human PSR (Cascade Bioscience, Winchester, MA) over night at 4°C. Biotinylated anti-mouse antibody was used as a secondary antibody (1:50) and subsequently visualized through fluorescein avidin (1:50; Vector Laboratories, Burlingame, CA).

Experimental Treatments. NO administration was performed by replacing the culture media with media containing 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine (NOC-9; 300 μM) (Calbiochem, San Diego, CA), 3-morpholinosydnonimine (SIN-1; 300 μM) (Calbiochem, San Diego, CA), or sodium nitroprusside (SNP; 300 μM) (Sigma, St. Louis, MO) per the experimental paradigm (Maiese and Vincent, 2000). More than one NO generator was used as a control to demonstrate that cells were responding to NO rather than to other by-products of these agents.

Assessment of Cell Survival. SH-SY5Y injury was determined by bright-field microscopy using a 0.4% trypan blue dye exclusion method 24 h after NO exposure per our previous protocols (Lin and Maiese, 2001). The mean survival was determined by counting eight randomly selected nonoverlapping fields, each containing approximately 10 to 20 cells (viable + nonviable). Each experiment was replicated four to six times independently with different cultures.

Assessment of DNA Fragmentation. Genomic DNA fragmentation was determined by the terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay (Lin and Maiese, 2001; Chong et al., 2002b). Briefly, SH-SY5Y cells were fixed in 4% paraformaldehyde/0.2% picric acid/0.05% glutaraldehyde, and the 3′-hydroxy ends of cut DNA were labeled with biotinylated dUTP using the enzyme terminal deoxytransferase (Promega, Madison, WI) followed by streptavidin-peroxidase and visualized with 3,3′-diaminobenzidine (Vector Laboratories).

Assessment of Membrane PS Residue Externalization. PS exposure was assessed through the established use of annexin V. Annexin V has been shown to strongly bind to membrane PS residues (Andree et al., 1990; Schutte et al., 1998; Hoffmann et al., 2001). In addition, annexin V binding is reversible during the chelation of calcium and the rates of association and dissociation demonstrate that annexin V does not penetrate cellular membranes (Andree et al., 1990; Vincent and Maiese, 1999a). Consequently, these features make annexin V an excellent tool for cellular membrane PS exposure identification.

Per our prior protocols (Maiese and Vincent, 2000; Lin and Maiese, 2001; Chong et al., 2002b), a 30 μg/ml stock solution of annexin V conjugated to phycoerythrin (R&D Systems, Minneapolis, MN) was diluted to 3 μg/ml in warmed calcium-containing binding buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$). Plates were incubated with 500 μl of diluted annexin V for 10 min. Images were acquired with "blinded" assessment with a Leitz DMIRB microscope (Leica, McHenry, IL) and a Fuji/Nikon Super charge-coupled device (6.1 megapixels) using transmitted light and fluorescent single excitation light at 490 nm and detected emission at 585 nm.

PI 3-K Inhibition of Akt1. PI 3-K inhibition was performed by administering wortmannin and LY294002 (Calbiochem, La Jolla, CA). Two structurally dissimilar PI 3-K inhibitors were employed to ensure that the biological effects observed were a result of PI 3-K inhibition. Wortmannin or LY294002 was added directly to the cultures 1 h before NO application and the treatment of PI 3-K inhibition was continuous.

Assessment of Mitochondrial Membrane Potential. The fluorescent probe JC-1 (Molecular Probes, Eugene, OR), a cationic membrane potential indicator, was used to assess the mitochondrial membrane potential. SH-SY5Y cells in 35-mm^2 plates were incubated with 2 μg/ml JC-1 in growth medium for 30 min. After washing, SH-SY5Y cells were then analyzed immediately under a Leitz DMIRB microscope (Leica, McHenry, IL) with a dual-emission fluorescence filter with 515 to 545 nm for green fluorescence and emission at 585 to 615 nm for red fluorescence.

Assessment of Cysteine Protease Activity. At specified times after NO exposure, cysteine protease activities were determined as described previously (Lin and Maiese, 2001; Chong et al., 2002b). Cell suspensions were prepared and an aliquot of supernatant containing 50 μg of protein was incubated with a 250 μM colorimetric substrate for caspase 3 (Ac-DEVD-pNA), caspase 8 (Ac-IETD-pNA), or caspase 9 (Ac-LEHD-pNA) (Calbiochem, San Diego, CA). Absorbance was measured at 405 nm and substrate cleavage was reported in micromoles per minute per gram of protein against standard *p*-nitroaniline solutions.

Modulation of Cysteine Protease Activity. Modulation of cysteine protease activity in SH-SY5Y cells was performed by using the irreversible and cell permeable caspase inhibitors (20 μM 1 h before NO) Z-DEVD-FMK (DEVD) for caspase 3, Z-IETD-FMK (IETD) for caspase 8, or Z-LEHD-FMK (LEHD) for caspase 9 (LEHD) (BD Biosciences Pharmingen, San Diego, CA).

Western Blot Analysis for Akt1 Phosphorylation, Cytochrome *c* Release, and Caspase Proteolytic Processing. Cells were homogenized and after protein determination, each sample (50 μg/lane) was then subjected to 7.5% (Akt1) or 12.5% (cytochrome *c*, caspase 3, and caspase 9) SDS-polyacrylamide gel electrophoresis. After transfer, the membranes were incubated with primary mouse monoclonal antibodies against phosphorylated Akt1/PKBα (p-Akt1, Ser 473; Upstate Biotechnology), cytochrome *c* (1:2000; BD Biosciences Pharmingen), or rabbit polyclonal antibodies against caspase 3 [H-277 (detection for p11, p17, and p20 subunits and the full-length precursor of caspase 3), 1:200] and caspase 9 [H-83 (detection for p10 subunit and the full-length precursor of caspase 9), 1:200] (Santa Cruz Biotechnologies, Santa Cruz, CA). After washing, the membranes were incubated with a horseradish peroxidase conjugated secondary antibody [goat anti-mouse IgG (1:2000) or goat anti-rabbit IgG (1:15,000) (Pierce, Rockford, IL)]. The antibody-reactive bands were revealed by chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Preparation of Mitochondria and Cytosol for the Analysis of Cytochrome *c* Release. Briefly, cells were harvested, homogenized, and the harvested supernatants were centrifuged at 10,000 g for 15 min at 4°C. The resulting pellet was resuspended in isolation buffer and used as the mitochondrial fraction. The supernatant was subjected to further ultracentrifugation at 50,000*g* for 1 h, with the resultant supernatant being used as the cytosolic fraction.

Statistical Analysis. For each experiment involving assessment of SH-SY5Y cell survival, DNA degradation, membrane PS exposure, microglial activation, mitochondrial membrane potential, and caspase activity, the mean and S.E. were determined from four to six replicate experiments. Statistical differences between groups were assessed by means of analysis of variance with the post hoc Student's *t* test. Results are expressed as the mean ± S.E. Statistical significance was considered at $p < 0.05$.

Results

Akt1 Maintains Cellular Integrity and Genomic DNA during NO Exposure. In Fig. 1, SH-SY5Y cells were exposed to the NO donor NOC-9 (300 μM) and 24 h later, cell survival was assessed by trypan blue, and cell DNA fragmentation was determined by TUNEL. Untreated wild-type control cells are without trypan blue staining or DNA fragmentation. In wild-type cells exposed to NO, cell injury is evident by decreased cell density, permeability to trypan blue dye, and chromatin condensation. In contrast, cells with stable myr-Akt1 overexpression exposed to NO were with significantly reduced trypan blue staining and nuclear condensation. Data for all stable expressing clones with a transfection efficiency equal to or greater than 98% (highest expressing

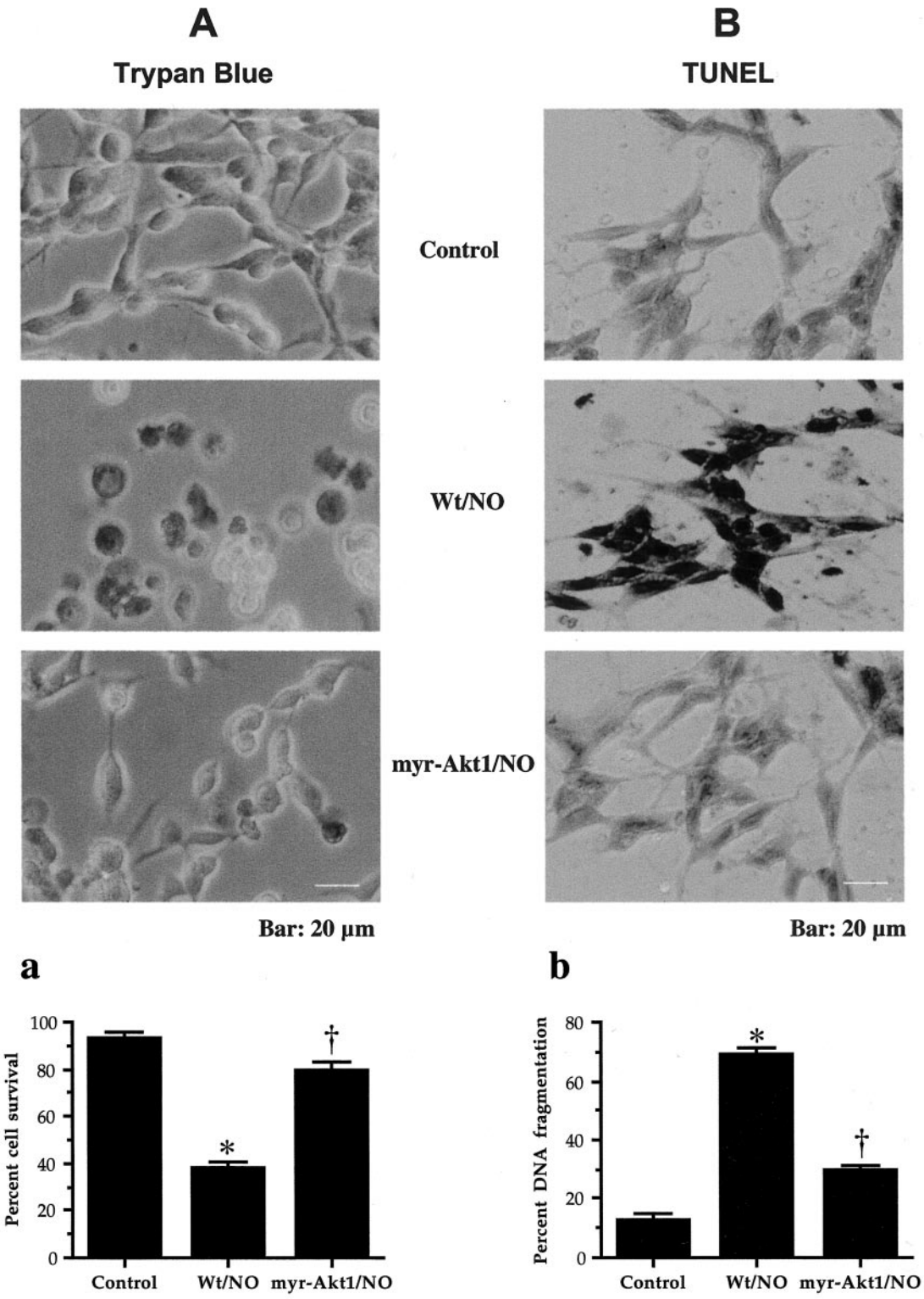

Fig. 1. Akt1 maintains cellular integrity and genomic DNA during NO exposure. Representative images illustrate cell survival with a trypan blue dye exclusion (A) and DNA fragmentation with TUNEL (B) in both wild-type (Wt) and myr-Akt1 transfected SH-SY5Y (myr-Akt1) cells 24 h after exposure to a NO donor (NOC-9, 300 μM). NO-induced cellular injury was evident in wild-type cells (Wt/NO), whereas there was no injury evident in Akt1-transfected cells (myr-Akt1/NO). a, cell survival in cells with stable transfection of myr-Akt1 was significantly increased compared with wild-type cells after NO exposure (*, $p < 0.01$, Wt/NO versus control; †, $p < 0.01$, myr-Akt1/NO versus Wt/NO). b, DNA fragmentation in cells with stable myr-Akt1 transfection was significantly less than that of wild-type cells after NO exposure (*, $p < 0.01$, Wt/NO versus control; †, $p < 0.01$, myr-Akt1/NO versus Wt/NO). To simplify a and b, data for the three NO donors (NOC-9, SIN-1, or SNP) were combined. In all cases, each data point represents the mean and S.E.M. Control, cultures without NO exposure.

clones resulting from selection methods) were combined because no significant differences in cell injury or DNA fragmentation were present among the individual clones with stable myr-Akt1 overexpression.

To quantitatively determine the ability of Akt1 to prevent NO-induced injury and DNA fragmentation, a NO donor (NOC-9, SIN-1, or SNP, 300 μM) was directly applied to wild-type cells and cells with stable myr-Akt1 overexpression and assessment was performed 24 h later. In Figs. 1, a and b, data for the three NO donors was combined because no significant differences in cell injury were present among the agents.

In wild-type SH-SY5Y cells, cell survival was significantly reduced from 93 ± 3% (untreated control cells) to 38 ± 3% (NO, 300 μM, $p < 0.01$) (Fig. 1a). By comparison, cells that actively overexpress myr-Akt1 significantly increased survival during NO exposure to approximately 80%. As shown in Fig. 1b, NO alone resulted in a significant increase in the percentage of DNA fragmentation (69 ± 2%) in wild-type cells compared with untreated control cultures (13 ± 2%). DNA fragmentation was reduced to 30 ± 1% in cells with stable myr-Akt1 overexpression after NO exposure.

Activation of the Akt1 Pathway Is Necessary and Sufficient for Cellular Protection During NO Exposure. Because myr-Akt1 overexpression can significantly enhance cell survival and limit genomic DNA fragmentation, we initially examined whether activation of Akt1 through its phosphorylation by PI 3-K was required to protect cells against toxic injury. Western blot assay was performed for phosphorylated Akt1 (p-Akt1) 12 h after NO exposure. In Fig. 2A, increased expression of p-Akt1 in wild-type cells and maintenance of increased p-Akt1 expression in cells with stable myr-Akt1 overexpression was present after NO exposure. This increased expression of p-Akt1 was decreased in both wild-type cells and cells with stable myr-Akt1 overexpression by the agents wortmannin (0.5 μM) and LY294002 (10 μM), specific inhibitors of PI 3-K phosphorylation of Akt1.

In Fig. 2B, application of wortmannin (0.5 μM) or LY294002 (10 μM) in wild-type cells reduced survival from 37 ± 3% in cells treated with NO alone to approximately 19 ± 2% in sister cultures, suggesting that a baseline activity of Akt1 in wild-type cells provides some level of protection during injury. In addition, administration of wortmannin (0.5 μM) or LY294002 (10 μM) in cells with stable myr-Akt1 overexpression reduced cell survival from 83 ± 5% in cells treated with NO alone to 61 ± 4% (Wort, 0.5 μM) and to 60 ± 4% (LY, 10 μM). When administered in the absence of NO, wortmannin (0.5 μM) and LY294002 (10 μM) were not toxic to cells (Fig. 2B). These results suggest that in either wild-type cells or cells with stable myr-Akt1 overexpression, an additional endogenous reserve of Akt1 protein exists to protect against cell injury.

To independently assess whether activation of Akt1 was necessary and sufficient for cellular protection, we examined whether the overexpression of a kinase-deficient, dominant-negative Akt1 in SH-SY5Y cells would alter cellular survival during NO exposure. Initially, Western blot assay was performed for p-Akt1 12 h after NO exposure in cells that overexpress dn-Akt1 (Fig. 2C). Similar to our previous results, increased expression of p-Akt1 in wild-type cells was present after NO exposure. In contrast, cells with dn-Akt1 overexpression that lacked kinase activity were without expression of p-Akt1.

To subsequently assess whether Akt1 was necessary for the prevention of NO-induced injury, a NO donor (NOC-9, SIN-1, or SNP, 300 μM) was directly applied to wild-type cells and cells with kinase deficient dn-Akt1 overexpression and assessment was performed 24 h later (Fig. 2D). In wild-type cells, cell survival was significantly reduced from 91 ± 3% (untreated control cells) to 39 ± 5% (NOC-9, 300 μM, $p < 0.01$), 40 ± 4% (SIN-1, 300 μM, $p < 0.01$), and 37 ± 5% (SNP, 300 μM, $p < 0.01$). This cell injury was significantly enhanced in cells that actively overexpress dn-Akt1 with significantly decreased survival during NO exposure to approximately 23%. These results suggest that activation of Akt1 is critical for protection during free radical exposure.

Akt1 Inhibits Microglial Activation, Proliferation, and PSR Expression. Because Akt1 offers intrinsic cytoprotection through the maintenance of intact genomic DNA, we next investigated whether Akt1 fostered extrinsic cellular protection through the prevention of microglial activation and proliferation. In Fig. 3A, representative microglial cultures illustrate a marked induction of microglial activation and proliferation during treatment with media from wild-type cells, as evidenced by significant PCNA (Williams et al., 2002) and BrdU expression (Martinez-Contreras et al., 2002). To a similar degree, treatment of microglia cultures with NO-exposed media from wild-type cells had a significant increase in PSR expression compared with microglia treated with media not exposed to NO (control). In contrast, minimal activation or proliferation of microglia as well as expression of the PSR is present during treatment with media from cells overexpressing myr-Akt1. In Fig. 3B, quantitation of PCNA, BrdU, and PSR labeling revealed that a significant expression in PCNA (67 ± 4%; 1.01 ± 0.06 × 10^5 cells/35-mm plate), BrdU (55 ± 2%; 0.83 ± 0.03 × 10^5 cells/35-mm plate), and PSR (75 ± 2%; 1.13 ± 0.03 × 10^5 cells/35-mm plate) was present in microglia cultures after the application of NO-treated media compared with untreated control cultures (28 ± 3%, PCNA, 0.42 ± 0.05 × 10^5 cells/35-mm plate; 18 ± 3%, BrdU, 0.27 ± 0.05 × 10^5 cells/35-mm plate; 39 ± 1%, PSR, 0.59 ± 0.02 × 10^5 cells/35-mm plate). In contrast, application of media from cells with overexpression of myr-Akt1 during NO exposure resulted in significantly less microglial activation with reduced PCNA expression (39 ± 2%, 0.59 ± 0.03 × 10^5 cells/35-mm plate), reduced BrdU uptake (30 ± 2%, 0.45 ± 0.03 × 10^5 cells/35-mm plate), and reduced PSR expression (52 ± 4%, 0.78 ± 0.06 × 10^5 cells/35-mm plate).

Membrane PS Exposure Is Necessary for Microglial Activation and Proliferation. Because externalization of membrane PS residues in injured neurons undergoing apoptosis can result in phagocytic elimination of these cells (Rucker-Martin et al., 1999; Maiese and Vincent, 2000; Hoffmann et al., 2001), we investigated whether externalization of membrane PS residues on SH-SY5Y cells was necessary and sufficient for the activation of microglia. In Fig. 4A, application of PS yielded a significant increase in PCNA expression (69 ± 3%), BrdU uptake (57 ± 1%), and PSR expression (77 ± 4%) compared with untreated control cultures (26 ± 2%, PCNA; 19 ± 4%; BrdU; 39 ± 2%, PSR). This activation and proliferation of microglia was specific for PS, because administration of

phosphatidylcholine (PC), a structurally related principal constituent of cell membranes but a biologically distinct membrane phospholipid used as an experimental control, did not significantly alter PCNA, PSR, or BrdU compared with untreated control microglial cultures.

Exposure of PS residues also was found to be both necessary and sufficient to induce microglial activation and proliferation. Administration of an antibody to PSR (PSR Ab) alone in a series of concentrations of 0.001 to 1.00 μg/ml did not alter microglial activation compared with untreated control cultures (Fig. 4B). However, specific antagonism against the microglial PSR receptor with the PSR Ab during the application of PS could prevent microglial activation as evidenced by the significant decrease in the expression of PCNA and the uptake of BrdU (Fig. 4C). Furthermore, in the presence of the PSR Ab during the application of NO-exposed media, the concentrations of PSR Ab of 0.10 and 1.00 μg/ml significantly decreased the capacity of NO to induce microglial activation, yielding values for PCNA (35 $\pm$ 2%) and BrdU (26 $\pm$ 3%) that were significantly less than values observed with NO-treated media in the absence of the PSR Ab (67 $\pm$ 5%, PCNA; 54 $\pm$ 3%; BrdU) (Fig. 4D). Taken

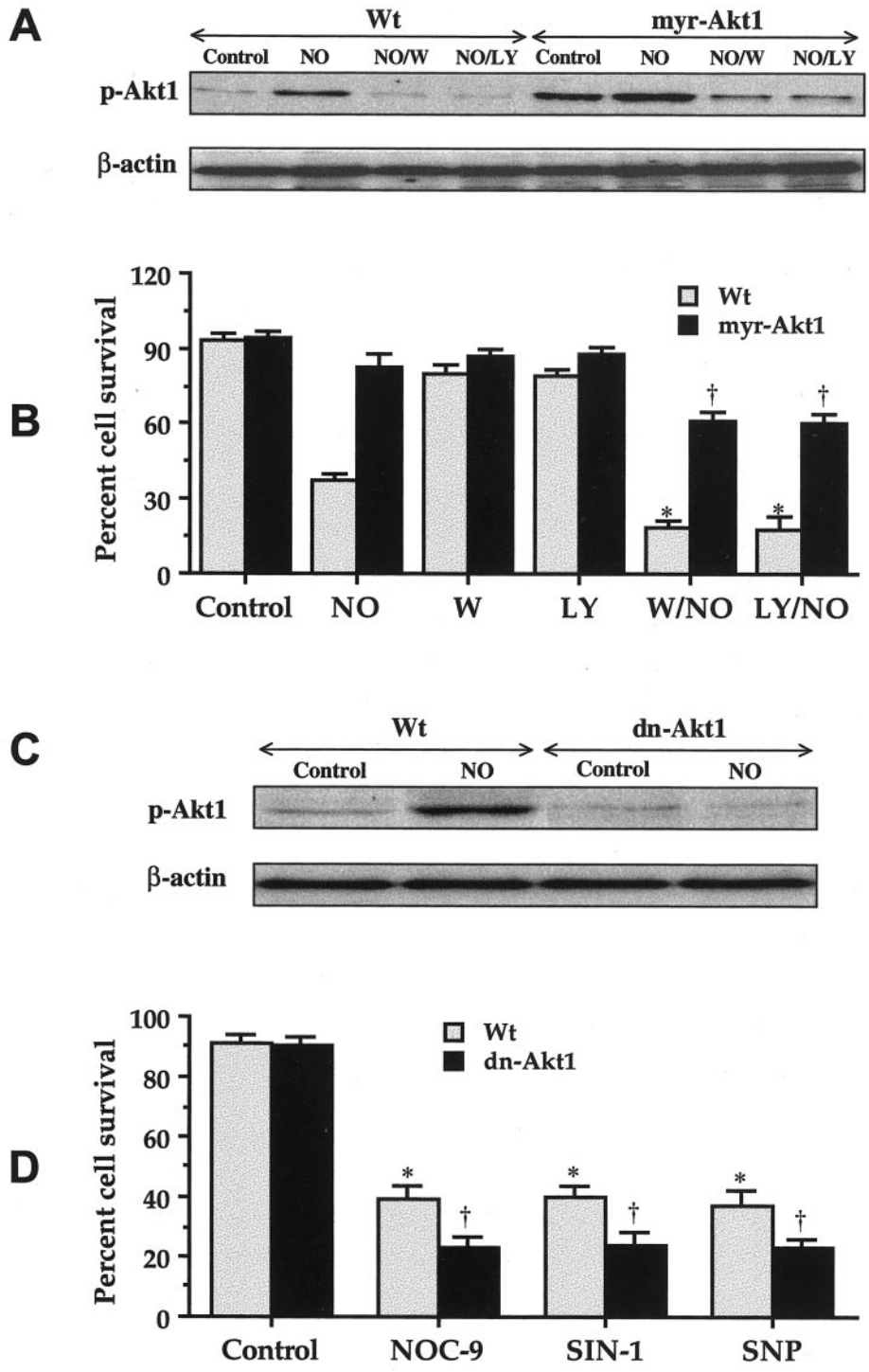

Fig. 2. Activation of Akt1 is necessary and sufficient for cellular protection. A, a representative Western blot illustrates that p-Akt1 expression is increased 12 h after NO exposure (NOC-9, 300 μM) in wild-type (Wt) cells or maintained in cells with myr-Akt1 overexpression but decreased by a 1-h pretreatment with wortmannin (W; 0.5 μM) or LY294002 (LY; 10 μM). Control, cultures without NO exposure. B, cell survival (trypan blue dye assay) in both wild-type (Wt) and cells with myr-Akt1 overexpression was determined 24 h after exposure to a NO donor (NOC-9, SIN-1 or SNP, 300 μM) in the presence of a 1-h pretreatment with either wortmannin (W; 0.5 μM) or LY294002 (LY; 10 μM). When applied alone, wortmannin (0.5 μM) or LY294002 (10 μM) were not toxic. In both wild-type (Wt) cells and cells with myr-Akt1 overexpression treated with wortmannin (0.5 μM) or LY294002 (10 μM), cell survival was significantly decreased compared with cells treated with NO alone (*, $p < 0.01$, Wt/NO versus Wt/Wort/NO or Wt/LY/NO; †, $p < 0.01$, myr-Akt1/NO versus myr-Akt1/Wort/NO or myr-Akt1/LY/NO). Control, cultures without NO exposure. Each data point represents the mean and SEM. To simplify the figures, data for the three NO donors (NOC-9, SIN-1 or SNP, 300 μM) were combined. C, a representative Western blot illustrates that p-Akt1 expression in wild-type cells is increased 12 h after NO exposure (NOC-9, 300 μM), but p-Akt1 expression is not present in cells with kinase-deficient, dn-Akt1 transfection. Control, cultures without NO exposure. D, cell survival in cells with dn-Akt1 transfection was significantly decreased compared with wild-type cells after exposure to a NO donor (NOC-9, SIN-1, or SNP, 300 μM) (*, $p < 0.01$ Wt/NO versus control; †, $p < 0.01$, dn-Akt1/NO versus Wt/NO). Control, cultures without NO exposure. Each data point represents the mean and S.E.M.

together, these results support the premise that modulation of microglial activation and proliferation by Akt1 during NO exposure is dependent upon inhibition of membrane PS exposure.

Akt1 Enhances Cell Survival through the Modulation of Caspase 3-, Caspase 8-, and Caspase 9-Like Activities. In Fig. 5, A–C, data for caspase 3-, caspase 8-, and caspase 9- like activities were obtained 6 and 12 h after NO exposure; these time periods represented the peak activities for these cysteine proteases (Lin and Maiese, 2001; Chong et al., 2002b). In Fig. 5A, the cleavage of Ac-DEVD-pNA, a substrate for caspase 3, was significantly increased from 0.11 ± 0.01 mmol/min/g in untreated wild-type cultures to 0.31 ± 0.02 μmol/min/g (6 h) and from 0.09 ± 0.01 μmol/min/g to 0.35 ± 0.03 μmol/min/g (12 h) after NO exposure. Stable expression of myr-Akt1 prevented the proteolytic processing of procaspase 3 and significantly decreased caspase 3 activity at 6 h (0.11 ± 0.01 μmol/min/g) and 12 h (0.21 ± 0.03 mmol/min/g) after NO exposure ($p < 0.01$) (Fig. 5A). Similarly, an increase in caspase 8-like activity (Fig. 5B) and caspase 9-like activity (Fig. 5C) was observed at 6 and 12 h after NO exposure in wild-type cultures. Stable expression of myr-Akt1 prevented significantly reduced the activity of caspase 8-like activity (0.12 ± 0.02 μmol/min/g; 12 h) and caspase 9-like activity (0.11 ± 0.02 μmol/min/g; 12 h) compared with wild-type cultures treated with NO alone (0.29 ± 0.02 μmol/min/g and 0.41 ± 0.02 μmol/min/g, 12 h, respectively). Among the three caspases, the ability of Akt1 to prevent procaspase 9 processing and block caspase 9-like activity seemed to be the most robust.

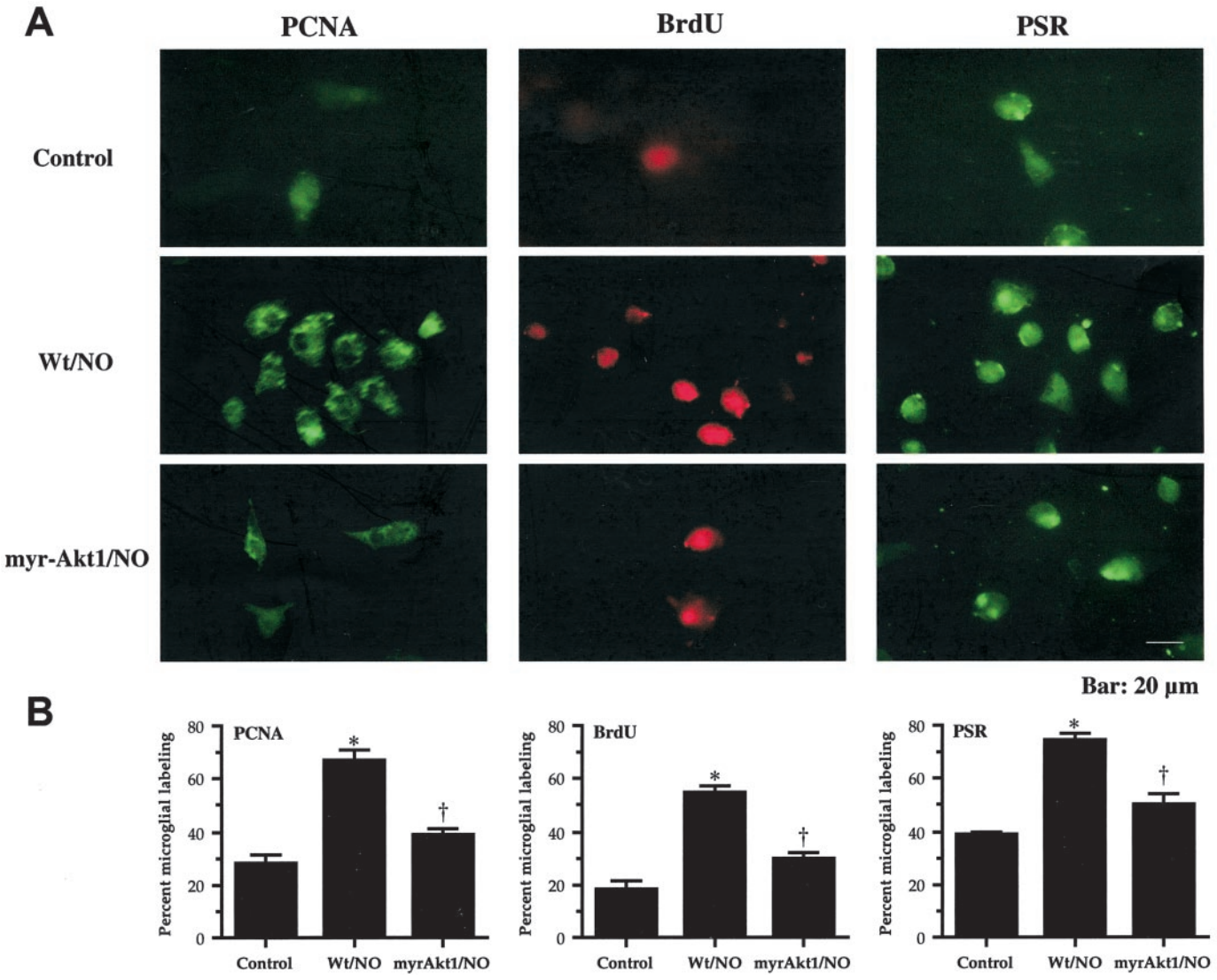

Fig. 3. Akt1 prevents microglial activation, proliferation, and PSR expression. Twenty-four hours after exposure to NO (NOC-9, SIN-1, or SNP, 300 μM), media from wild-type cultures (Wt/NO) and cells overexpressing myr-Akt1 (myr-Akt1/NO) was applied to pure cultures of microglia for 3 h. Twelve hours later, microglial activation was assessed through PCNA and PSR expression. Microglial proliferation was observed with BrdU. A, representative images illustrates that PCNA, PSR, or BrdU expression was significantly increased in microglia treated with media from NO-exposed wild-type cells (Wt/NO). In contrast, PCNA expression, PSR expression, or BrdU uptake was significantly less in microglia treated with media from NO-exposed cells with myr-Akt1 overexpression (myr-Akt1/NO). B, PCNA expression, PSR expression, or BrdU uptake in microglia treated with media from NO-exposed wild-type cells (Wt/NO) were significantly increased, but PCNA, PSR expression, or BrdU uptake were significantly diminished in microglia treated with media from NO-exposed cells with myr-Akt1 overexpression (myr-Akt1/NO) (*, $p < 0.01$, Wt/NO versus control; †, $p < 0.01$, myr-Akt1/NO versus Wt/NO). To simplify the figures, the results of the three NO donors (NOC-9, SIN-1, or SNP, 300 μM) were combined. Control, cultures without NO exposure.

We next examined whether the induction of caspase 3-, caspase 8-, and caspase 9-like activities were required for NO -induced cell injury. As shown in Fig. 5D, wild-type cells exposed to NO (NOC-9, SIN-1, or SNP, 300 μM) resulted in a cell survival of 37 ± 5%. Pretreatment of SH-SY5Y cells with 20 μM of DEVD, IETD, and LEHD to inhibit caspase 3-, caspase 8-, and caspase 9-like activities significantly increased cell survival to approximately 71 ± 5, 70 ± 5, and 73 ± 6%, respectively.

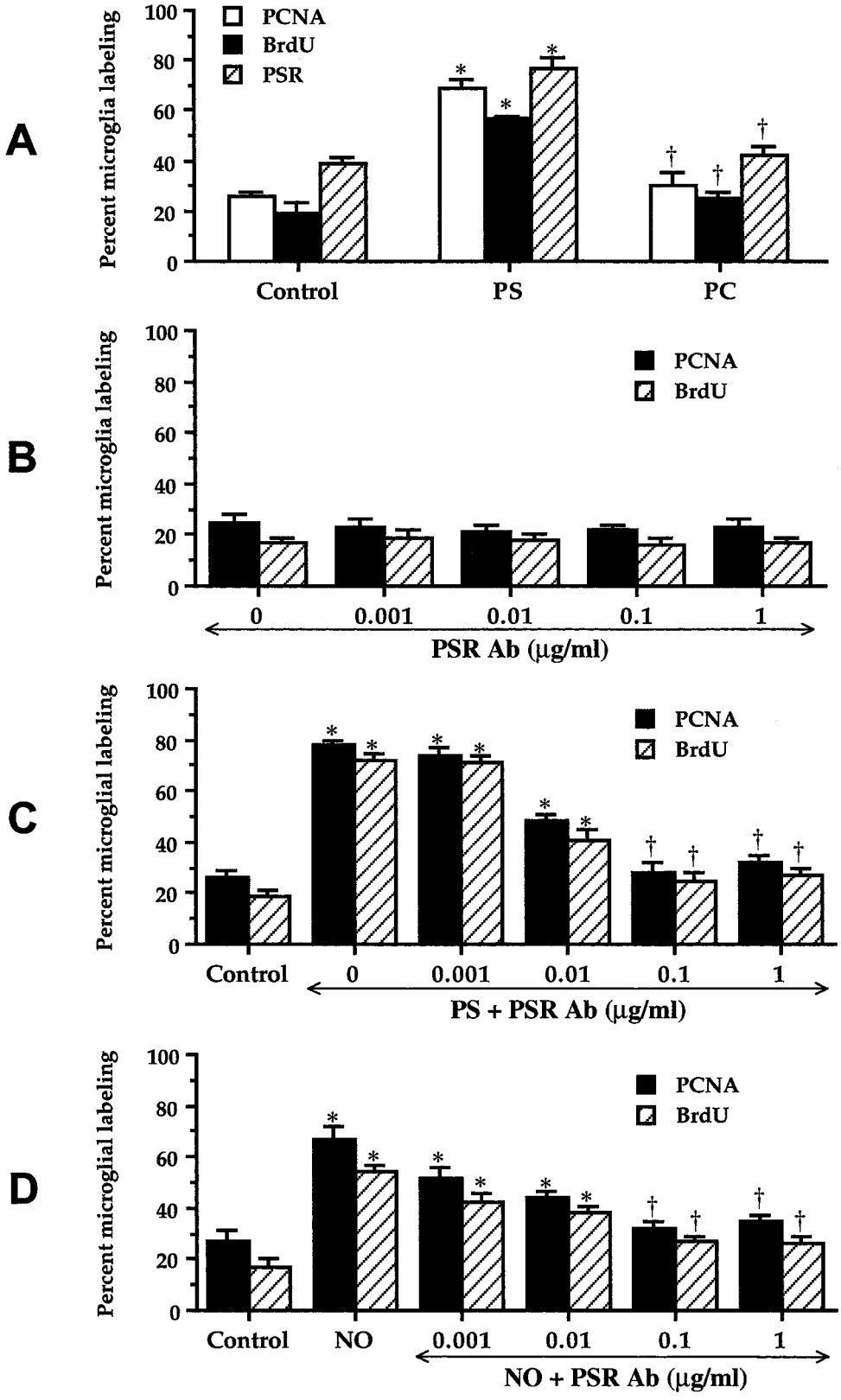

Fig. 4. Membrane PS exposure is necessary for microglial activation and proliferation. A, media containing PS (10 μM) or PC (10 μM) was applied to pure cultures of microglia for 3 h. PC, a principal constituent of cell membranes but a biologically distinct membrane phospholipid, was used as an experimental control. Twelve hours later, microglial activation and proliferation were assessed through PCNA expression, PSR expression, and BrdU uptake. Activation and proliferation of microglia were specific for PS, because administration of PC did not significantly alter PCNA, PSR, or BrdU compared with untreated control microglial cultures. (*, $p < 0.01$, PS or PC versus control). Control, cultures without PS or PC exposure. B, administration of an antibody to PSR (PSR Ab) alone in a series of concentrations of 0.001 to 1.00 μg/ml did not alter microglial activation (PCNA) or proliferation (BrdU) compared with untreated control cultures. C, media containing PS (10 μM) was applied to pure cultures of microglia for 3 h treated with increasing concentrations of the PSR Ab (0.001–1 μg/ml). Twelve hours later, microglial activation was assessed through PCNA expression and BrdU uptake. Specific antagonism against the microglial PSR receptor with the PSR Ab during the application of PS prevented microglial activation and proliferation. Control, untreated microglial cultures. D, 24 h after exposure to NO (NOC-9, SIN-1, or SNP, 300 μM), media from wild-type cultures was applied to pure cultures of microglia for 3 h treated with increasing concentrations of the PSR Ab (0.001–1 μg/ml). Twelve hours later, microglial activation was assessed through PCNA expression and BrdU uptake. Specific antagonism against the microglial PSR receptor with the PSR Ab during the application of NO-exposed media blocked microglial activation and proliferation. Control, untreated microglial cultures. To simplify the figures, data for the three NO donors (NOC-9, SIN-1, or SNP) were combined. In all cases, each data point represents the mean and S.E.M.

Akt1 Prevents Microglial Activation Primarily through Membrane PS Exposure and the Inhibition of Caspase Activity. We next assessed whether Akt1 prevented microglial activation through the modulation of membrane PS exposure. We initially examined the ability of Akt1 to modulate externalization of membrane PS exposure in SH-SY5Y cells during NO exposure. In Fig. 6A, SH-SY5Y cells were exposed to the NO donor NOC-9 (300 μM); 24 h later, cell membrane PS exposure was determined by annexin V. Untreated wild-type control cells were without annexin V label. In wild-type cells exposed to NO, there was evidence of marked induction of annexin V label. In contrast, cells with stable myr-Akt1 overexpression exposed to NO were with significantly less annexin V label. To further quantitatively determine the ability of Akt1 to prevent NO-induced membrane PS exposure, a NO donor (NOC-9, SIN-1, or SNP, 300 μM) was directly applied to wild-type and myr-Akt1 overexpression cells and assessment was performed over a 24-h period (Fig. 6B). A progressive increase in annexin V label was observed in wild-type cells at 4 and 24 h after exposure to a NO donor (NOC-9, SIN-1, or SNP, 300 μM) that reached a maximum of 66 $\pm$ 1% compared with untreated control cultures of 11 $\pm$ 4%. Cells with stable myr-Akt1 overexpression displayed a significant reduction in annexin V label to 21 $\pm$ 1% and 30 $\pm$ 2% at 4 and 24 h after NO exposure.

Because the cysteine proteases caspase 3 and caspase 9 have been linked to the externalization of membrane PS residues (Vanags et al., 1996; Lin and Maiese, 2001; Mandal et al., 2002), we examined whether the ability of Akt1 to directly modulate caspase 3- and caspase 9-like activities was biologically relevant for the maintenance of cellular membrane asymmetry. We quantitated the ability of each caspase to modulate membrane PS exposure in SH-SY5Y cells and subsequent microglial activation and proliferation (Figs. 6, C and D). In Fig. 6C, NO exposure (NOC-9, SIN-1, or SNP, 300

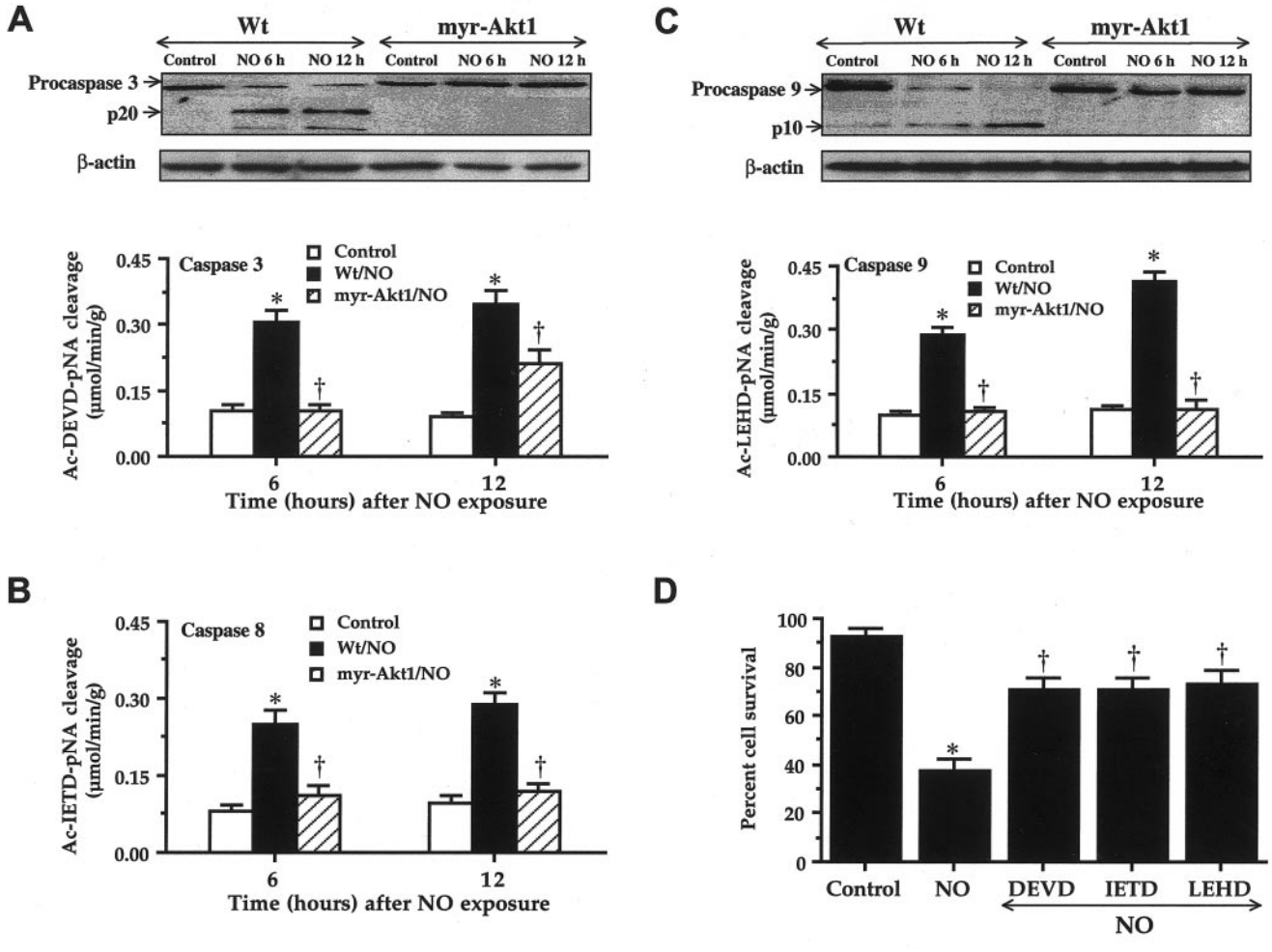

Fig. 5. Akt1 prevents cellular injury through the modulation of caspase 3-, caspase 8-, and caspase 9-like activities. Wild-type (Wt) and myr-Akt1 transfected cells (myr-Akt1) were exposed to a NO donor (NOC-9, SIN-1, or SNP, 300 μM) and caspase 3- (A), caspase 8- (B), and caspase 9- (C) like activities were determined at 6 and 12 h later through their respective colorimetric substrates. In addition, proteolytic processing of procaspase 3 (A) and procaspase 9 (C) with their respective cleaved products were assessed through immunoblotting at 6 and 12 h later after NO exposure. Stable expression of myr-Akt1 prevented the proteolytic processing of procaspase 3 (A) and procaspase 9 (C) and significantly inhibited the increase in the activity of caspase 3 (A), caspase 8 (B), and caspase 9 (C) induced by NO exposure (*$p < 0.01$, Wt/NO versus control; †, $p < 0.01$, myr-Akt1/NO versus Wt/NO). Each data point represents the mean and S.E.M. Control, cultures without NO exposure. D, wild-type cells were pretreated with a caspase 3 inhibitor (DEVD, 20 μM), caspase 8 inhibitor (IETD, 20 μM), or a caspase 9 inhibitor (LEHD, 20 μM) 1 h before a NO donor (NOC-9, SIN-1, or SNP, 300 μM) and survival was determined 24 h after NO (*$p < 0.01$, NO versus control; †, $p < 0.01$, DEVD/NO, IETD/NO, LEHD/NO versus NO). In all cases, control cultures are those without NO exposure, and data for the three NO donors (NOC-9, SIN-1, or SNP, 300 μM) were combined to simplify the figure.

μM) in wild-type SH-SY5Y cells resulted in an annexin V label of 63 ± 2%. The inhibition of each of the caspases significantly decreased annexin V label to 45 ± 2% (caspase 3) and 42 ± 2% (caspase 9). In Fig. 6D, a significant expression in PCNA (69 ± 4%), BrdU (52 ± 5%), and PSR (77 ± 2%) was present in microglia cultures with NO-treated media compared with untreated control cultures (27 ± 2%, PCNA; 19 ± 4%, BrdU; 38 ± 2%, PSR). Similar to our results with caspase inhibition of membrane PS exposure in wild-type SH-SY5Y cells, inhibition of each of the caspases significantly decreased microglia expression of PCNA, PSR, and BrdU.

Modulation of Caspase Activity by Akt1 May Be Dependent Upon Mitochondrial Membrane Depolarization and the Release of Cytochrome c. Because activation of specific caspases may be initiated through mitochondrial membrane depolarization and cytochrome *c* release (Li et al., 1997; Yabuki et al., 2000), we examined the ability of Akt1 to alter mitochondrial membrane potential after exposure to NO. Exposure to NO (NOC-9, SIN-1, or SNP, 300 μM) produced a significant decrease in the red/green fluorescence intensity ratio using a cationic membrane potential indicator JC-1 within 3 h compared with untreated control cells (Fig. 7A), suggesting that NO results in mitochondrial membrane depolarization. Stable expression of activated Akt1 during NO exposure significantly increased the red/green fluorescence intensity of SH-SY5Y cells, indicating that mitochondrial permeability

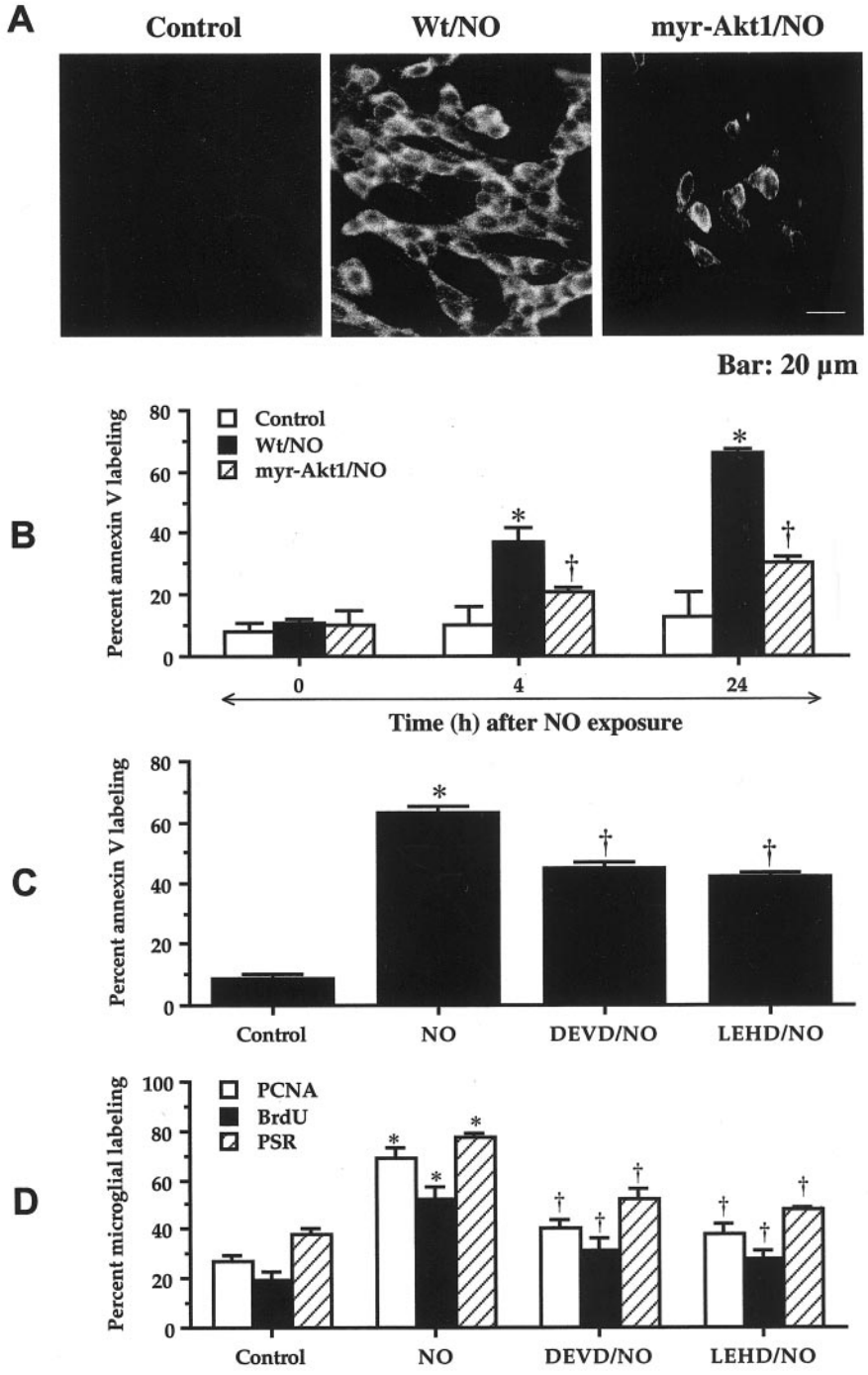

Fig. 6. Akt1 prevents microglial activation primarily through membrane PS exposure and the inhibition of caspase activity. A, representative images illustrate membrane PS externalization with annexin V phycoerythrin to visualize PS externalization in both wild-type (Wt) and cells with myr-Akt1 overexpression (myr-Akt1) cells 24 h after exposure to a NO donor (NOC-9, 300 μM). Membrane PS exposure in injured cells is evident by yellow fluorescent staining (Wt/NO) with no significant membrane PS exposure evident in myr-Akt1 transfected cells (myr-Akt1/NO). B, membrane PS exposure in cells with myr-Akt1 overexpression was significantly reduced at 4 and 24 h after NO exposure compared with wild-type cells (*, $p < 0.01$, Wt/NO versus control; †, $p < 0.01$ myr-Akt1/NO versus Wt/NO). Control, cultures without NO exposure. C, membrane PS exposure in cells during caspase 3 (DEVD, 20 μM) or caspase 9 (LEHD, 20 μM) inhibition was significantly reduced at 24 h after NO exposure compared with wild-type cells (*, $p < 0.01$, NO versus control; †, $p < 0.01$ DEVD/NO, LEHD/NO versus NO). D, media from wild-type cultures that was exposed to a 24-h application of NO (NOC-9, SIN-1, or SNP, 300 μM) and a specific caspase inhibitor [caspase 3 (DEVD, 20 μM) or caspase 9 (LEHD, 20 μM)] per the experimental paradigm was applied to pure cultures of microglia for 3 h. Twelve hours later, microglial activation was assessed through the PCNA, PSR, and BrdU. Inhibition of each of the caspases significantly decreased microglia expression of PCNA, PSR, and BrdU (*, $p < 0.01$, NO versus control; †, $p < 0.01$, YVAD/NO, DEVD/NO, LEHD/NO versus NO). To simplify B, C, and D, data for the three NO donors were combined. Each data point represents the mean and S.E.M. Control, cultures without NO exposure.

transition pore membrane potential was restored to baseline. In addition to maintaining mitochondrial permeability transition pore function, stable expression of Akt1 prevented mitochondrial cytochrome *c* release into the cytosol, as demonstrated by Western analysis (Fig. 7B).

Discussion

For strategies to be efficacious against neurodegenerative processes, they must focus upon both intrinsic pathways of neuronal integrity as well as extrinsic pathways of cellular disposal. In this respect, we have identified Akt1 as a neuroprotectant that holds a novel position to not only preserve cellular survival and genomic DNA integrity but also to modulate apoptotic membrane PS exposure in conjunction with microglial activation and proliferation. We subsequently elucidated several key cellular mechanisms that were necessary for the execution of both intrinsic and extrinsic cellular pathways to assist in the promotion of cell survival.

Through the overexpression of a kinase-deficient, dominant-negative Akt1, we demonstrate that Akt1 is both necessary and sufficient to protect cells from NO-induced injury. Cells with dominant-negative overexpression that lacked kinase activity were without expression of p-Akt1 and suffered a significant loss in cell survival during NO exposure. These results illustrate that Akt1 provides a necessary and sufficient level of protection during free radical injury. Interestingly, we also have found that a cellular reserve of Akt1 exists in either wild-type cells or in cells with stable myr-Akt1 overexpression that is amenable to phosphorylation to assist in the promotion of cell survival. During NO exposure, inhibition of PI 3-K phosphorylation of Akt1 with wortmannin or LY294002 reduced survival in wild-type cells, suggesting that phosphorylation and activation of endogenous Akt1 during cellular injury provides at least a minimal level of protection. This "reserve" for cellular protection by Akt1 continues to exist also in cells with stable myr-Akt1 expression. Prevention of phosphorylated Akt1 using the PI 3-K inhibitors wortmannin or LY294002 in cells with stable myr-Akt1 overexpression further limits cytoprotection. In essence, without this induction of phosphorylated Akt1 during toxic exposure, cell survival would be significantly worsened. This work illustrates that endogenous activation and phosphorylation of Akt1 can provide an additional level of protection and functions in concert with the exogenous activation of

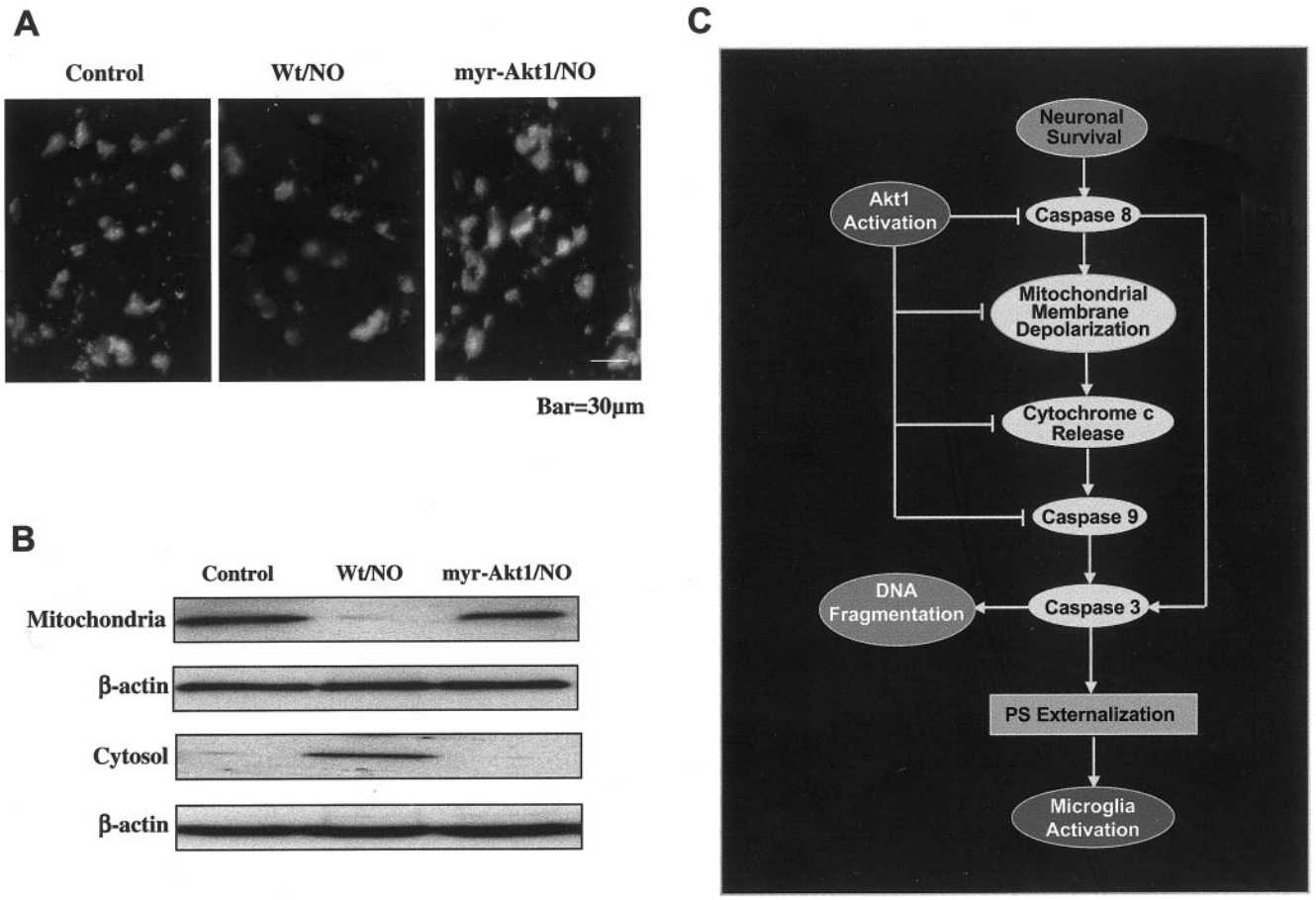

Fig. 7. Akt1 prevents mitochondrial membrane depolarization and cytochrome *c* release. A, mitochondrial staining with a membrane potential indicator (JC-1; 2 μg/ml, 30 min) was performed in both wild-type cells (Wt/NO) and cells with myr-Akt1 overexpression (myr-Akt1/NO) 3 h after exposure to NO (NOC-9, 300 μM). Stable expression of myr-Akt1 prevented mitochondrial depolarization. B, a representative Western blot with equal amounts of mitochondrial protein and cytosol extracts (50 μg/lane) illustrates that cells with myr-Akt1 overexpression (myr-Akt1/NO) significantly prevents cytochrome *c* release from mitochondria to the cytosol during NO exposure compared with wild-type (Wt/NO) cells. In all cases, control cultures are those without NO exposure. C, Akt1 possesses the novel ability to maintain cellular integrity and prevent cellular removal through inhibition of microglial activation and proliferation. Akt1 prevents DNA degradation through a series of cellular pathways that involve mitochondrial membrane polarization, cytochrome *c*, and inhibition of caspase 3, caspase 8, and caspase 9. In addition, Akt1 maintains cellular membrane asymmetry through caspase 3 and caspase 9 inhibition, which protects against microglial activation with subsequent phagocytic destruction of cells.

Akt1 (cells with stable myr-Akt1 overexpression) to achieve a higher level of cell protection.

Akt1 offers intrinsic cytoprotection not only through the maintenance of intact genomic DNA but also through extrinsic mechanisms by inhibiting cellular membrane PS exposure. In several cell systems, membrane PS externalization functions to identify cells that have entered the early stages of apoptosis and to expedite the elimination of these cells through phagocytosis (Rucker-Martin et al., 1999; Maiese and Vincent, 2000; Hoffmann et al., 2001). Exposure of membrane PS residues, even in cells that have undergone repair and are without further injury, can promote cell-to-cell interactions and lead to the "tagging" of cells for removal by microglia that require increased PSR expression on their cell surface (Maiese and Vincent, 2000; Hoffmann et al., 2001). Prevention of membrane PS exposure can provide an additional mechanism to avert cell disposal and cell loss.

Our present work provides further insight into the ability of Akt1 to protect cells from inflammatory injury and phagocytic removal through the exposure of membrane PS externalization. First, we illustrate that microglial activation and proliferation, as assessed through PCNA expression, PSR expression, and BrdU uptake, occurs during NO-mediated cellular injury. Second, we demonstrate that exposure of membrane PS residues (detected by annexin V label) by cells undergoing apoptosis is both necessary and sufficient to induce microglial activation and proliferation, because application of an antibody to the PSR prevents microglial activation and proliferation during NO-mediated injury. Prior studies have demonstrated that during apoptotic injury, cells can shed annexin V-binding membrane particles that are complementary to membrane PS residues during apoptotic cellular injury (Simak et al., 2002). Third, we show that media taken from cells that overexpress myr-Akt1 during NO exposure leads to a significant reduction in the expression of PCNA, the expression of PSR, and the expression of BrdU. Taken together, our work demonstrates that Akt1 provides a novel level of protection against cellular membrane PS exposure and the possible shedding of membrane PS particles during free radical injury. Furthermore, the studies provide strong evidence that modulation by Akt1 of cellular membrane PS exposure and complementary microglial activation is biologically relevant and enables Akt1 to offer a unique level of extrinsic cellular protection through the prevention of cellular inflammation and neuronal phagocytic disposal.

A series of cellular pathways that may be intricately linked to one another seem to be responsible for cytoprotection by Akt1. At one level, cellular integrity and membrane PS exposure that is tied to microglial activation is closely associated with the induction of cysteine protease activity. The ability of Akt1 to modulate caspase 3-, caspase 8-, and caspase 9-like activities seems to play a critical role in the protection conferred by Akt1. These cysteine proteases are associated with the independent apoptotic pathways of genomic DNA cleavage and cellular membrane PS exposure (Takahashi et al., 1999; Lin and Maiese, 2001). Caspase 9 is activated through a process that involves the cytochrome *c*-Apaf-1 complex (Li et al., 1997; Chong et al., 2002a). In addition, caspase 8 serves as an upstream initiator of executioner caspases, such as caspase 3, and also leads to the mitochondrial release of cytochrome *c* (Engels et al., 2000; Stegh et al., 2002). After caspase 8 and caspase 9 activation, caspase 3 leads directly to genomic DNA degradation. Experimental models that use caspase 3 gene deletions or pharmacological inhibition illustrate little or no DNA fragmentation after toxic cellular insults (Keramaris et al., 2000; Lin and Maiese, 2001). Our data also suggest that Akt1 prevents membrane PS exposure through the inhibition of caspase 9 and 3-like activities. Caspase 3 is believed to be responsible for the externalization of membrane PS residues in several cell systems through the digestion of cytoskeletal proteins, such as fodrin, and to be responsible for microglial phagocytosis (Vanags et al., 1996; Maiese and Vincent, 2000). Our present work further supports the premise that the downregulation of caspase 3- and 9-like activities by Akt1 is tied to the direct activation and proliferation of microglia.

Another cellular pathway that seems closely associated with the ability of Akt1 to prevent cysteine protease activity is the modulation of mitochondrial membrane potential. Mitochondria-mediated apoptosis can be initiated by free radical injury and results in the cytoplasmic release of cytochrome *c* (Bal-Price and Brown, 2000; Chong et al., 2002a). In our present studies, we demonstrate that the free radical NO leads to the depolarization of the mitochondrial membrane in cells with the subsequent release of cytochrome *c* into the cytosol. Consistent with other studies that demonstrate preserved mitochondrial function as a result of Akt activation during irradiation injury (Kennedy et al., 1999), our current work further elucidates the cellular mechanisms of Akt1 to prevent free radical induced injury by demonstrating that overexpression of myr-Akt1 directly maintains mitochondrial membrane potential and prevents the release of cytochrome *c*.

In conclusion, we illustrate that Akt1 provides a unique level of cytoprotection that addresses both intrinsic pathways of cellular integrity and extrinsic mechanisms that involve cell removal through phagocytosis (Fig. 7C). Cellular protection by Akt1 is both necessary and sufficient to foster the maintenance of both genomic DNA integrity and cellular membrane asymmetry. Akt1 maintains nuclear DNA integrity through the specific inhibition of caspase 3-, 8-, and 9-like activities that are linked to the mitochondrial release of cytochrome *c*. In addition, apoptotic neuronal membrane PS exposure provides a novel mechanism for Akt1 to offer extrinsic cellular protection and block microglial activation.

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