Akt Activation Protects Hippocampal Neurons from Apoptosis by Inhibiting Transcriptional Activity of p53*

Received for publication, September 19, 2000, and in revised form, October 25, 2000
Published, JBC Papers in Press, October 27, 2000, DOI 10.1074/jbc.M008552200

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Survival factors suppress apoptosis by activating the serine/threonine kinase Akt. To investigate the molecular mechanism underlying activated Akt’s ability to protect neurons from hypoxia or nitric oxide (NO) toxicity, we focused on the apoptosis-related functions of p53 and caspases. We eliminated p53 by employing p53-deficient neurons and increased p53 by infection with recombinant adenovirus capable of transducing p53 expression, and we now show that p53 is implicated in the apoptosis induced by hypoxia or NO treatments of primary cultured hippocampal neurons. Although hypoxia and NO induced p53, treatment with insulin-like growth factor-1 significantly inhibited caspase-3-like activation, neuronal death and transcriptional activity of p53. These insulin-like growth factor-1 effects are prevented by wortmannin, a phosphatidylinositol 3-kinase inhibitor. Adenovirus-mediated expression of activated-Akt kinase suppressed p53-dependent transcriptional activation of responsive genes such as Bax, suppressed caspase-3-like protease activity and suppressed neuronal cell death with no effect on the cellular accumulation and nuclear translocation of p53. In contrast, overexpression of kinase-defective Akt failed to suppress these same activities. These results suggest a mechanism where Akt kinase activation reduces p53’s transcriptional activity that ultimately rescues neurons from hypoxia- or NO-mediated cell death.

The p53 tumor suppressor gene has recently been shown to play an important role in regulating apoptosis for neurons and a number of other cell types (1). Hypoxic insult, as well as excitotoxic stimulation, involves excessive production of nitric oxide (NO) and has been shown to increase p53 production with concomitant increase in neuronal apoptosis (2). In general, p53-mediated apoptosis requires a p53 protein that functions to modulate transcription of certain genes. Important examples of such activated genes include the apoptosis-inducing Bax gene (3) and the KILLER/DR5 gene (4).

Following exposure to toxic treatments, a number of peptide factors including neurotrophins and growth factors such as insulin-like growth factor 1 (IGF-1)* have been found to enhance neuronal survival (5). In recent years, considerable advances have been made in understanding the signal transduction pathway activated by these growth factors. Among them, the serine/threonine kinase termed Akt appears to play a central role in the survival of a number of cell types (6–9). Recently, four Akt substrates were identified that are also components of the cell’s intrinsic death machinery: the proapoptotic Bcl-2 family member known as Bad (10, 11), the caspase-9 protease (12), IκB kinase α (IKKα) (13), and a member of the Forkhead family of transcription factors known as FKHL1 (14–16). In each case, phosphorylation by Akt kinase inhibits these protein’s pro-apoptotic function, thereby accounting for at least part of the survival effect imparted by activated Akt kinase.

We now report that activated Akt significantly impairs p53-dependent neuronal apoptosis and impairs induction of Bax in response to treatment with hypoxia, NO, or adenovirus-mediated expression of p53. We further show that activated Akt strongly inhibits p53-dependent transactivation of target genes with no apparent effect on p53-protein accumulation, subcellular localization, and phosphorylation status of p53. These findings suggest a mechanism where activated Akt kinase inhibits p53-mediated transactivation to apparently block subsequent apoptosis of the neuron.

EXPERIMENTAL PROCEDURES

p53-deficient Mice—Homozygous p53-deficient mice were obtained from the Oriental Yeast Co., Ltd. (Tokyo, Japan). Originally on a mixed C57BL6/CBA strain, p53-deficient mice were backcrossed to C57BL6 (five times) so that the majority of their genetic background is C57BL6. Fifth generation heterozygous p53 knockout mice on the C57BL6 background were mated to one another to produce the homozygous p53 knockout mice used in this study. The genotypes of all mice were confirmed by PCR using DNA extracted from the tails according to the protocol described previously (17). The primers were mixtures of 1) a genomic sequence in intron 1 and upstream of exon 2 in the p53 gene (5′-AATTTGACAAATTTAGTCATAACATTAGACTA-3′), 2) a genomic sequence in exon 4 of the p53 gene (5′-ACTCTTTACATCTGCGGGCAGCAGCAAGATG-3′), and 3) a sequence from the neomycin resistance gene (5′-GAACCTGCGTGCTGCAATTCAACTATCTTGGGAGATG-3′). A 500-base pair PCR product represents the wild-type p53 gene and a 800-base pair PCR product represents the disrupted p53 gene.

Cell Culture—Hippocampal neurons were prepared from postnatal day 1–p53 wild-type and deficient mice and from embryonic day 18

* This work was supported by Core Research for Evolutional Science and Technology of Japan Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: IGF-1, insulin-like growth factor-1; Ad, adenovirus; m.o.i., multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; RT, reverse transcriptase; PCR, polymerase chain reaction; DIV, days in vitro; CMV, cytomegalovirus; AMC, 7-amino-4-methylcoumarin; SNP, sodium nitroprusside.
Harlan Sprague Dawley rat embryos, as described previously (18, 19).

Briefly, fetal hippocampi were dissected and digested with 0.25% trypsin for 20 min at room temperature in calcium/magnesium-free Hanks' balanced salt solution (Life Technologies, Inc.). Tissues were further dissociated by repeated trituration. The cells were seeded at a density of 1 × 10^6 cells/10 cm plates on coverslips (10 cm) (Falcon Lab and Maintainware, Lincoln Park, NJ) and grown in Dulbecco's modified Eagle's medium supplemented with 10% inactivated fetal calf serum, 30 mM glucose, and 0.5% (v/v) penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ and 95% room air. To inhibit growth of glial cells, cytosine arabinoside (10 μM) was added to the culture media after seedling. All experiments were performed in 8–10-day-old cultures (8–10 DIV).

Experimental Treatments—The growth medium was replaced with a serum-free medium that consisted of Dulbecco's modified Eagle's medium supplemented with 30 mM glucose and 0.5% (v/v) penicillin-streptomycin. At 24 h after the replacement, cells were treated for 6 h with IGF-1 or vehicle (saline) and exposed to hypoxia or a NO donor, sodium nitroprusside (SNP), or were untreated as stated. Cells were incubated at 37 °C for 24 h before exposure to hypoxia or a NO donor, incubated with virus-containing medium. The cells were incubated with virus-containing medium at the indicated multiplicity of infection at 37 °C for 60 min. Titers were determined by plaque assay and concentrated virus stored in fresh media at the indicated multiplicity of infection at 37 °C for 24 h. (24 h later), neurons were suspended in buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 10 mM EGTA), and then incubated with 10 μM digitiom (Sigma) at 37 °C for 10 min, followed by centrifugation at 15,000 rpm for 3 min. Protein concentration in the resulting supernatant was measured using a DC protein assay kit (Bio-Rad). Then, the supernatant containing 30 μg of protein was incubated with 50 μM enzyme substrate, 7-amino-4-methylcoumarin (AMC) (DEVD), at 37 °C for 1 h. Levels of released AMC were measured using an excitation wavelength of 380 nm and an emission wavelength of 460 nm with a spectrofluorometer (Hitachi F-3000, Hitachi, Tokyo, Japan). One unit was defined as the amount of enzyme required to release 0.22 nmol of AMC/min at 37 °C. Caspase-3-like activity of control neurons was 47.1 ± 2.7 units/mg of protein.

Western Blot Analysis—Cell extracts for Western blot analysis were prepared by washing the cells three times with PBS and lysing them in sample buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1% SDS, and 100 mM NaCl). The samples were boiled for 5 min before subjecting 20-μg aliquots to electrophoresis on 12.5% SDS-PAGE gels. After the proteins were transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), the membrane was incubated in blocking buffer (1× PBS, 5% nonfat dried milk) for 1 h at room temperature and then probed with a primary antibody in blocking buffer overnight at 4 °C. The membrane was washed four times in PBS containing 0.3% Tween 20, probed with the secondary antibody in blocking buffer for 1 h at room temperature, and washed again in PBS containing 0.05% Tween 20. Detection of signal was performed with an enhanced chemiluminescence detection kit (Amersham International, Little Chalfont, United Kingdom). The primary antibodies used were anti-p53 monocular antibody (DO-1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Bax polyclonal antibody (P-19; Santa Cruz Biotechnology, Inc.), anti-mouse Akt polyclonal antibody (New England Biolabs), anti-mouse phospho-Akt polyclonal antibody that recognizes Akt only when phosphorylated at Ser-473 (9270, New England Biolabs), and anti-mouse β-actin monoclonal antibody (Sigma).

RNA Isolation and RT-PCR—Total RNA from 2 × 10⁶ cells was extracted from primary cultures of rat neurons by the acid guanidium-phenol chloroform method. For RT-PCR, 5 μg of total RNA was reverse-transcribed using oligo(dT) and reverse transcriptase (Promega). The resulting cDNA was used as a template. PCR amplification was performed using specific oligonucleotide primer pairs (10 pmol each) in a volume of 25 μl. For PCR analysis, specific oligonucleotide primer pairs (10 pmol each) were incubated with 1 μl of cDNA template in a 20-μl PCR reaction mixture containing 1.5 mM MgCl₂, 25 mM KCl, 10 mM Tris, pH 9.2, 1 μl of deoxynucleotides (1 mM each), and 1 unit of Taq polymerase. The sequences of primers used in this study were as follows: Bax sense primer (5′-TGTTGCCTTCTCTACTTGTGG-3′) and Bax antisense primer (5′-TGGAGGAGGATCGT-3′); and, for β-actin, the primer pair was 5′-ACTACGCTATGAGTCTGGC-3′ and 5′-TCGGTTCATTGTGGGCACT-3′. Dilutions of cDNA were amplified for 23–26 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The amplified PCR products at each cycle number were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The product of constitutively expressed β-actin mRNA served as the internal standard. The amplified PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The product of constitutively expressed β-actin mRNA served as the control. All the products were assayed in the linear response range of the RT-PCR amplification process; the cycle number used was determined by finding the midpoint of linear amplification on a sigmoid curve for both amplification products with cycle numbers of 24–30 plotted against band density (25). The identity of each PCR product was confirmed by subcloning the amplified cDNAs into the pGEM-T vector (Promega) and sequencing.

Electrophoretic Mobility Shift Assay (EMSAs)—Nuclear extracts were prepared from primary neuronal cultures according to published method (26) with some modifications. In brief, cells were plated at a density of 10⁶ cells/60-mm dishes and were treated with 3 mM hydrogen peroxide for 5 min and washing in 0.5 ml of cold PBS. The cells were then washed once in 0.1 mM of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM dithiothreitol). The washed pellets were then suspended in 0.1 μl of buffer A plus 0.1% Nonidet P-40 supplemented with 1 μM leupeptin and aprotinin and were incubated on ice for 10 min. After incubation, the pellets were mixed briefly by vortexing and were cen-
trifuged at 10,000 rpm at 4 °C for 5 min in a microcentrifuge. The supernatant was carefully removed, and the nuclear pellet was resuspended in 20 μl of cold buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.15 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) containing 1 μg/ml leupeptin and aprotinin and incubated on ice for another 15 min with intermittent vortexing. The extracts were then centrifuged at 10,000 rpm at 4 °C for 10 min, and the supernatant was divided into aliquots and frozen at -70 °C. Protein concentrations were determined using Bio-Rad protein assay kit.

To assay binding activity, a double-strand oligonucleotide containing the sequence corresponding to the p53 consensus site (5'-AGGGTACA- CATCGCTAGATGCTGTT-3') (27) was end-labeled with [γ-32P]ATP using T4 kinase (Life Technologies, Inc.). Unincorporated nucleotides were removed using Sephadex G-50 column (Amersham Pharmacia Biotech). Binding reactions were carried out in a final volume of 25 μl consisting of 10 mM HEPES, pH 7.9, 4 mM Tris-HCl, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1.5 mg/ml bovine serum albumin, 2 μg of poly(dI-dC), 2–10 μg of nuclear extract, and 0.5 ng of 32P-labeled oligonucleotide probe (50,000 cpm). Reactions were incubated for 20 min at room temperature. Binding reactions were subjected to nondenaturing polyacrylamide electrophoresis through 4% gels in a 1X Tris borate-EDTA buffer system. Gels were dried and subjected to autoradiography.

Luciferase Assay—Primary cultured rat neurons were plated in six-well tissue culture dishes at 9 × 10⁵ cells/well and used for transient transfection at DIV 7. Cells were then cotransfected with 0.3 pmol of a p53-Luc plasmid that contains a firefly luciferase reporter gene driven by a basic promoter element and a TATA box, which are joined to a tandem repeat of a p53 binding element (Stratagene), together with 0.3 pmol of pRL-TK plasmid, which contains an herpes simplex virus thymidine kinase promoter upstream of the Renilla luciferase gene (Promega). As controls, pGL3 basic vector or pGL3 promoter plasmids that contain an SV40 promoter upstream of the firefly luciferase gene (Promega) are cotransfected with pRL-TK plasmid. Transfections are carried out using the modified calcium phosphate method as described previously (28).

Transfected cells were cultured for 24 h, washed twice with 2 ml of Ca²⁺- and Mg²⁺-free PBS and lysed with Passive Lysis Buffer (Promega). Firefly luciferase and Renilla (sea pansy) luciferase activities were measured sequentially using a dual-luciferase reporter assay system (Promega) and a Lumat LB9501 luminometer (EG&G, Berthold). After measuring the firefly luciferase signal (Lₐ) and the Renilla luciferase signal (Lₐₐ), the relative luciferase activity (RLA) was calculated as RLA = Lₐₐ/Lₐ, where relative RLA was calculated as a percentage, i.e. %RLA = RLA/RLₐₐₐₐ.

In Vivo Kinase Assay—To examine the effect of Akt activation on the phosphorylation status of p53, in vivo kinase assays were performed according to the published method (29). Neurons derived from rat fetal brains were seeded in 35-mm dishes at the density of 1–2 × 10⁶ cells/dish. Cells were incubated in a phosphate-free medium for 30 min and then labeled with [32P] orthophosphate (0.02 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and phosphatase inhibitors (50 mM NaF, 1 mM Na₃VO₄). p53 proteins were recovered by immunoprecipitation with anti-p53 antibody and subjected to SDS-PAGE, which was dried prior to analysis by autoradiography.

Statistical Analysis—Results are presented as mean ± S.E. Experimental groups were compared by analysis of variance, followed by Scheffé’s post hoc test. p values less than 0.05 were considered significant.

RESULTS

p53 Is Involved in Caspase-3-like Activity and Neuronal Death Mediated by Hypoxic and Nitric Oxide Treatments—To address the possibility that p53 plays a role in cell death following treatment with hypoxia or NO donor, SNP, we compared viabilities of primary hippocampal neurons derived from wild-type or p53-null mice. Treatment with hypoxia or SNP (50 μM) induced significantly greater degrees of neuronal loss, DNA fragmentation, and caspase-3-like activation in neurons derived from wild-type mice as compared with those from p53-null mice (Fig. 1A, c–f). Administration of the Ac-DEVD-CHO, a peptide inhibitor of caspase-3-like protease (1 μM), which completely inhibited caspase-3-like activation (Fig. 1A, c), reduced the proportion of hypoxia-induced or SNP-induced cell death (Fig. 1A, b) and DNA fragmentation (Fig. 1A, a), suggesting that increased protease activity is required for cell death. Furthermore, treatment with hypoxia or SNP significantly increased the levels of p53 protein (Fig. 1A, d). These results demonstrated that apoptosis following hypoxia or SNP treatments is prominently associated with increased p53 expression.

To further demonstrate that p53 is involved in neuronal apoptosis, we used a strategy of adenovirus infection to transduce the expression of p53 protein in cultures of hippocampal neurons from p53-null mice. Western blot analysis revealed that cultured neurons deficient in p53 gene and infected with Ad5CMVp53, which expresses human wild-type p53 protein under control of the CMV promoter, displayed increased levels of p53 protein at 24 h after infection that was dependent on the multiplicity of infection (m.o.i.) as compared with uninfected cells or with cells infected with a control virus, AxCANLZ (Fig. 1B, a). Infection with Ad5CMVp53 (50 and 100 m.o.i.) also increased neuronal death, DNA fragmentation, and caspase-3-like activity at 24 h after infection (Fig. 1B, b–d). Moreover, pretreatment of neurons with the caspase-protease inhibitor, Ac-DEVD-CHO (1 μM), significantly inhibited neuronal death induced by infection with Ad5CMVp53 (Fig. 1B, c). DNA fragmentation, neuronal viability, and caspase-3-like proteolytic activity of AxCANLZ-infected cells were similar to those of control, uninfected neurons (data not shown). These results are consistent with the hypothesis where increased levels of p53 expression are proportionally associated with increased apoptotic cell death.

IGF-1-mediated Signaling Pathways Inhibit p53-induced Neuronal Death—Growth factors such as IGF-1 appear to play a neuroprotective role against ischemic or excitotoxic insult (30). To assess the role of IGF-1 in the prevention of p53-mediated neuronal death, we examined the effect of IGF-1 on wild-type or p53-null neurons exposed to hypoxia or SNP treatment. Pretreatment with IGF-1 conferred ~70% protection from hypoxia- or SNP-induced cell death, 60% reduction in DNA fragmentation, and 70% inhibition of caspase-3-like activation in wild-type neurons with these effects being blocked by treatment with wortmannin (20 nM), an inhibitor of PI 3-kinase (Fig. 2, A–C). On the other hand, IGF-1 treatment in p53-null neurons did not significantly change the measured parameters of apoptosis observed in non-IGF-1-treated p53-null neurons (Fig. 2, A–C). These results clearly suggest that IGF-1 protects neurons from p53-mediated toxicity through PI 3-kinase activation.

IGF-1 Mediates Inhibited DNA Binding and Transcriptional Activity of p53 with No Effect on p53 Protein Accumulation—The functional status of p53 to activate transcription strongly correlates with its ability to bind specific DNA sequences on target genes (31). To examine the effect of IGF-1 on p53 accumulation, DNA binding activity, and transcriptional activity, primary rat hippocampal neurons were pretreated with or without IGF-1 in the presence or absence of wortmannin followed by exposure to hypoxia or SNP treatments for up to 24 h. 24 h after exposure to hypoxia or SNP, cells were lysed and the extracts were subjected to Western blot analysis using anti-p53 monoclonal antibody. As shown in Fig. 3A, IGF-1 treatment with or without pretreatment of wortmannin had no effect on p53 accumulation in neurons exposed to hypoxia or SNP treatment. We then examined the effect of IGF-1 on p53’s DNA binding activity by EMSAs. Nuclear extract were prepared 8 h after treatment with hypoxia or SNP, and the EMSAs were
performed using the p53 consensus response element as a probe (5'-AGCTTAGACATGCCTAGACATGCCTA-3'). As shown in Fig. 3B, treatment with IGF-1 inhibits hypoxia- or SNP-induced increases in p53's DNA binding activity. Pretreatment with wortmannin apparently antagonized the IGF-1 effect and allowed increased p53 DNA binding activity. Excess unlabeled probe effectively competed the binding of the labeled probe (Fig. 3B). To examine whether IGF-1 affects the transactivation potential of p53, we performed reporter gene assays using reporter plasmids containing a tandem repeat of a p53 consensus response element placed upstream of a luciferase cDNA (p53-Luc, Stratagene). 24 h after transient transfection with the reporter plasmid, cells were exposed to hypoxia or SNP treatment for 8 h in the presence or absence of IGF-1. Although we observed 8–9-fold induction of the luciferase activity by hypoxia or SNP treatment, pretreatment with IGF-1 remarkably reduced the increase in the transcriptional activation of p53, which was prevented by wortmannin (Fig. 3C).

Activation of Akt Kinase Inhibits p53-mediated Neuronal Death—Akt, a downstream target of PI 3-kinase, has been implicated in transducing growth factor survival signals in various cell types including neurons (32). As we found that
IGF-1 inhibits p53-mediated neuronal death through activation of PI 3-kinase, we then assessed a potential function of Akt activation in the prevention of p53-mediated neuronal death using an adenoviral vector capable of expressing constitutively active Akt with the Src myristoylation signal fused in-frame to the c-Akt coding sequence (AxCALNLmyrAkt). As a control, we employed another vector capable of expressing a kinase-defective Akt mutant (AxCAAKT179M) in which the lysine of the adenosine triphosphate-binding site at position 179 was replaced by a methionine. Primary hippocampal neurons were infected with AxCANCre and either AxCALNmyrAkt or AxCALNLZ, a control virus carrying LacZ instead of myrAkt. Other cells were infected with AxCAAKT179M, and whole cell extracts were prepared for Western blot analysis 24 h after viral infection. Western blot analysis revealed increased levels of Akt and phosphorylated Akt in neurons infected simultaneously with AxCANCre (2 m.o.i.) and AxCALNmyrAkt (2 and 10 m.o.i.), in a m.o.i.-dependent manner (Fig. 4A). As the myrAkt lacks its pleckstrin homology domain, the size of the protein product (47.5 kDa) is smaller than endogenous Akt or

![Fig. 2. IGF-1 inhibits p53-induced neuronal death through PI 3-kinase.](image)

Neurons derived from wild-type (p53+/+) or p53-null mice (p53−/−) pretreated with IGF-1 (100 ng/ml) or vehicle (saline) in the presence or absence of wortmannin (W/20 nM) were exposed to hypoxia or SNP (50 μM) for 24 h, and then neuronal viability (A), DNA fragmentation (B), and caspase-3-like activity (C) were measured as described in text. Data are mean ± S.E. of four independent experiments performed in triplicates. *, *p < 0.05 versus non-IGF-1-treated p53+/+ neurons; #, *p < 0.05 versus IGF-1-treated p53+/+ neurons in the absence of wortmannin.

![Fig. 3. Effect of IGF-1 on cellular accumulation, DNA binding, and transcriptional activity of p53.](image)

Cultured rat hippocampal neurons pretreated with IGF-1 (100 ng/ml) or saline (vehicle) in the presence or absence of wortmannin (W; 20 nM) were exposed to hypoxia or SNP (50 μM). A, Western blot analysis. Extracts of neurons were subjected to 10% SDS-PAGE. Then the blots were probed with antibody to p53. Visualization of proteins was performed with ECL. B, EMSAs. Nuclear extracts prepared from neurons exposed to hypoxia or SNP for 8 h were assayed by EMSAs. “Cold” means the addition of excess of unlabeled oligonucleotide containing p53 consensus binding element into binding reactions as indicated -fold. The arrowhead points to the p53/p53 consensus binding element oligonucleotide complex. C, reporter gene assays. Primary hippocampal neurons were transiently cotransfected with reporter construct either p53-Luc plasmid or control plasmid, together with pRL-TK plasmid, and then neurons were treated with IGF-1 in the presence or absence of wortmannin. After 24 h of treatments, cultures were exposed for 8 h to hypoxia or SNP (50 μM) and luciferase activity was assayed. Relative luciferase activity was expressed as -fold increase, compared with control LacZ-expressing neurons without any stimuli (Control). Data are mean ± S.E. of four independent experiments performed in triplicate. *, *p < 0.05 versus nontreated neurons.
Akt Activation and p53-mediated Neuronal Death

Translocation, Phosphorylation Status, DNA Binding Activity, and Transcriptional Activation—Since we show that IGF-1 reduces the DNA binding activity and transcriptional activity of p53 without changing p53 protein accumulation through PI 3-kinase (Fig. 3, A–C), we further tested whether Akt activation inhibits changes in p53 accumulation, nuclear translocation, DNA binding activity, and transcriptional activity that are induced by hypoxia treatment, SNP treatment, or Adp53 infection. Cells infected with both AxCANCRe (2 m.o.i.) and AxCALNLmyrAkt (10 m.o.i.) or AxCALNLNZ (10 m.o.i.) were exposed to hypoxia or SNP (50 μM) or infected with Ad5CMVp53 (50 m.o.i.). Cellular accumulation, nuclear translocation, and DNA binding activity of p53 were assessed at 24, 12, and 8 h, respectively, after exposure to hypoxia or SNP, and at 24 h after AdCMVp53 infection. Cellular accumulation and nuclear translocation of p53 were observed following all the treatments and were not changed by expression of active Akt, kinase-defective Akt, or LacZ (Fig. 5, A and B). However, EMSAs using the p53 consensus response element as a probe showed that expression of active Akt, but not of kinase-defective Akt or LacZ, inhibited the hypoxia-, NO-, or Adp53-induced increase in DNA binding activity of functional p53 to the consensus binding oligonucleotide (Fig. 5C). Consistent with this finding, reporter gene assays using reporter plasmids containing tandem repeats of p53 consensus response element placed upstream of a luciferase cDNA revealed that expression of activated Akt, but not of kinase-defective Akt or LacZ, significantly reduced the induction of the luciferase promoter that is controlled by the p53-responsive promoter following hypoxia, SNP, or Adp53 infection (Fig. 5D). Next, to examine the possibility that Akt directly or indirectly phosphorylates p53 and thereby regulates p53’s transcriptional activity, the phosphorylation status of p53 was assessed by an in vivo kinase assay. Rat primary cultured neurons were infected with both AxCANCRe (2 m.o.i.) and AxCALNLmyrAkt (10 m.o.i.) or AxCALNLNZ (10 m.o.i.) or infected with AxKDAkt (10 m.o.i.) alone. After 24 h of incubation, cells were infected with Ad5CMVp53 (50 m.o.i.) and incubated for an additional 24 h to overexpress p53. Then cells were incubated for 3 h in the medium containing 0.2 mCi/ml [32P]orthophosphate. The phosphorylation status of p53 was monitored by immunoprecipitating p53 protein and quantifying p53-associated radioactivity by autoradiography. p53 protein expression was monitored by Western blotting analysis. As shown in Fig. 5E, our in vivo kinase assay showed that neither expression of active or kinase-defective Akt nor LacZ, significantly inhibits up-regulation of Bax mRNA levels following hypoxia, SNP, or Adp53 infection (Fig. 5E).

Akt Activation Inhibits p53-mediated Expression of Bax, a Proapoptotic Gene—Previous studies have shown that the Bax genes contain p53 response elements in their promoter and that their expression is transcriptionally regulated by functional p53 (3). As a member of the Bcl-2 family, Bax is required for p53-mediated cell death pathway in neurons (33, 34). Therefore, we examined whether Akt activation inhibits p53-mediated Bax expression. Cells were infected with both AxCANCRe (2 m.o.i.) and AxKDAkt (10 m.o.i.) alone and allowed to rest for 24 h before treatment with hypoxia, with SNP (50 μM), or with infection with Adp53 (50 m.o.i.) for another 24 h. Expression of active Akt, but not the kinase-defective form of Akt, significantly inhibited up-regulation of Bax mRNA levels following exposure of cells to hypoxia, to SNP or to infection with p53 adenovirus, as assessed by RT-PCR (Fig. 6A). Similar to increasing mRNA levels, Western blot analysis also revealed that expression of active Akt, but not kinase-defective Akt, blocked the p53-mediated increase of Bax protein levels (Fig. 6B).

Effect of Akt Kinase Activation on p53 Accumulation, Nuclear

Fig. 4. Activation of Akt kinase inhibits p53-induced neuronal death. A, assessment of adenovirus-mediated expression of active and kinase-defective forms of Akt. Cultured rat hippocampal neurons were infected with AxCANCRe (AxCre) and AxCALNLZ (AxLacZ) or AxCALNLmyrAkt (AxAkt), or AxCANCReK179M (AxKDAkt) at the indicated m.o.i. 24 h after infection, cell extracts were subjected to 10% SDS-PAGE and blots were probed with antibodies for Akt or phospho-Akt. B and C, cultures were infected with both AxCANCRe (AxCre; 2 m.o.i.) and AxCALNLNZ (AxLacZ; 10 m.o.i.) or AxCALNLmyrAkt (AxAkt; 10 m.o.i.), or AxCANCReK179M (AxKDAkt; 10 m.o.i.) alone. 24 h after infection, neurons were exposed for 24 h to hypoxia, 50 μM SNP or infected with Adp53 (50 m.o.i.). Then, the neuronal viability (B) and caspase-3-like activity (C) were assessed as described in the text. Data are mean ± S.E. of four independent experiments performed in triplicate. *, p < 0.05 versus neurons infected with both AxCre and AxLacZ in each treatment.

the product of kinase-defective Akt (62 kDa). The cells infected with AxCANCRe (2 m.o.i.) and AxCALNLZ (10 m.o.i.) did not show increased levels of Akt protein or phosphorylated Akt protein (Fig. 4A). Infection with AxCANCRe (2 m.o.i.) and AxCALNLmyrAkt (10 m.o.i.) to express activated Akt significantly reduced neuronal death and caspase-3-like activation induced by hypoxia, SNP, and p53 adenovirus infection compared with infection with AxKDAkt (10 m.o.i.) to express inactive, kinase-defective Akt protein (Fig. 4, B and C). Similarly, infection with AxCALNLNZ (10 m.o.i.) failed to reduce neuronal death compared with cells expressing activated-Akt kinase.
DISCUSSION

In the present study, we demonstrate that Akt can significantly inhibit p53-mediated neuronal death by reducing the ability of p53 to transactivate gene transcription. Previous data showed that p53 is up-regulated in neuronal cells in response to diverse insults such as ischemia, and Bax-mediated caspase-3 activation plays an important role in p53-induced apoptosis of neuronal cells (35). In this study, treatment of p53-null neurons with hypoxia or a NO donor turned out to reduce the induction levels of caspase-3-like activity, DNA fragmentation and neuronal death when compared with those of wild-type neurons. Exogenous adenovirus-mediated transduction of p53 expression in p53-deficient neurons up-regulated caspase-3-like activity and induced caspase-dependent DNA fragmentation. These results strongly suggest that p53 expression induced by treatments with hypoxia or a NO donor is responsible for a large portion of caspase-mediated apoptosis in primary hippocampal neurons.

A variety of mammalian cells are dependent on growth factors for their survival. Certain growth factors may also confer protection against apoptosis by stimulating PI 3-kinase and/or a downstream effector, the Akt kinase, in various cell types including neuronal cells (36). The present study showed that IGF-1 blocked p53-mediated caspase-3-like activation and cell death via PI 3-kinase. Adenovirus-mediated expression of active Akt inhibits caspase-3-like proteolytic activity and protects
neurons from apoptosis induced by hypoxia, SNP, or p53 adenovirus treatment. These results clearly indicate that activation of Akt is capable of suppressing the caspase-mediated neuronal death that is provoked by p53 expression.

How does Akt activation inhibit p53-mediated neuronal death? The final outcome of p53 activation depends upon many factors including the downstream targets associated with growth control and cell cycle checkpoints (p21, GADD45, MDM2), DNA repair (GADD45, p21, PCNA), and apoptosis (Bax, Bcl-x, Fas, IGF-BP) (37). Sequence-specific transactivation is required for p53-induced apoptosis in specific experimental systems (38). On the other hand, p53-mediated apoptosis does not necessarily require transcriptional activation (39). Here, we show that the p53 protein induced by treatments with hypoxia, SNP, or with p53 adenovirus infection appears to serve as a site-specific transcription factor that transactivates pro-apoptotic genes such as Bax. In addition, the increase in p53’s transcriptional activity was inhibited by expression of active Akt, but not of kinase-defective Akt. This observation was further confirmed by endogenous activation of Akt induced by IGF-1. Furthermore, p53-mediated Bax expression was also inhibited by expression of active Akt. Considering the previous observations that demonstrate a role of Bax in p53-mediated cell death (33, 34), our results suggest that activation of Akt kinase serves to protect neurons from apoptosis, at least in part, by inhibiting the transcriptional activity of p53.

There are several mechanisms that may explain how Akt suppresses transcriptional activity of p53. Several factors appear to contribute to the regulation of p53’s transcriptional activity including post-translational protein modification, status of protein conformation, p53 protein levels, and its cellular localization (40). In our experiments, activated Akt had no effect on the protein accumulation and nuclear translocation of p53. In addition, Akt activation has no effect on phosphorylation status of p53. Considering that essentially all activated Akt translocates into nucleus where it functions to phosphorylate proteins (41), these data suggest that Akt phosphorylates coactivators that directly or indirectly modify p53’s transcriptional activity, thereby reducing p53-mediated transcriptional activity and apoptosis. Additional experiments will be required to determine the precise mechanism by which activation of Akt kinase inhibits p53’s transcriptional activity.

In summary, we propose a novel mechanism in which activation of Akt inhibits p53-mediated neuronal death. More specifically, we report that Akt activation inhibits the transcriptional activity of p53, which we show leads to a reduction of Bax expression and a reduction in neuronal apoptosis. Thus, in addition to interfering with apoptotic events involving Bad and caspase-9, Akt activation appears to be capable of suppressing p53’s function at the level of p53-mediated transcription.

**Acknowledgment**—We thank Dr. Saito (University of Tokyo, Tokyo, Japan) for providing AdCMCcre, AdCMALNZ, and AdCMALNLz; Dr. Kikkawa (Kobe University, Kobe, Japan) for providing pT7-701 containing Akt1 K179M; and Dr. Roth (University of Texas, Houston, TX) for providing AdSCMVp53.

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