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Insulin-like Growth Factor-1 Protects Peroxynitrite-Induced Cell Death by Preventing Cytochrome *c*-Induced Caspase-3 Activation

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Abstract We investigated the effect of IGF-1 on cell death induced by peroxynitrite in human neuroblastoma SH-SY5Y cells. Exposure of the cells to 3-morpholinosydnonimine (SIN-1), a peroxynitrite donor, caused cytochrome *c* release from the mitochondria, caspase-3-like activation, and cell death. Pre-incubation of the cells with the caspase-3 inhibitor partially prevented SIN-1-induced cell death. Simultaneous addition of IGF-1 reduced SIN-1-induced caspase-3-like activation and cell death, whereas IGF-1 failed to reduce the release of cytochrome *c*. IGF-1 increased Akt phosphorylation, and Akt phosphorylation was inhibited by wortmannin, an inhibitor of phosphatidylinositol 3-kinase. In addition, wortmannin prevented IGF-1-evoked inhibition of cell death and caspase-3-like activation. In a cell-free system, addition of cytochrome *c* to cytosolic fraction resulted in caspase-3-like activation. The activation was reduced when the cytosolic fraction prepared from IGF-1-treated cells was used. These results suggest that IGF-1 protects peroxynitrite-induced cell death downstream of cytochrome *c* release through the inhibition of caspase-3-like activation. J. Cell. Biochem. 84: 708–716, 2002.

Key words: peroxynitrite; IGF-1; cytochrome *c*; caspase; cell death; SH-SY5Y

Insulin-like growth factor-1 (IGF-1) is a polypeptide hormone essential for the development of nervous system [Zackenfels et al., 1995]. IGF-1 is a potent mitogen and survival factor. For instance, IGF-1 promotes survival of cerebellar granule neurons against low potassium or serum deprivation [D'Mello et al., 1993], and prevents neuronal cell death induced by amyloid β-protein [Dore et al., 1997] or oxidative stress such as nitric oxide and hydrogen peroxide [Tamatani et al., 1998a; Heck et al., 1999]. Although IGF-1 has gained increasing attention for the treatment of neurodegenerative disorders, the precise mechanism by which IGF-1 prevents cell death is not fully understood. Phosphatidylinositol 3-kinase (PI3-kinase) is reported to be involved in IGF-1-mediated prevention of cell death [Yao and Cooper, 1995; Parrizas et al., 1997]. One of the downstream target molecules of PI3-kinase is a serine/threonine kinase, Akt [Dudek et al., 1997].

Extensive studies indicate that aspartate-specific cysteine proteases (caspases) are effectors of apoptosis [Yuan et al., 1993; Alnemri et al., 1996; Thornberry and Lazebnik, 1998]. Among them, caspase-3 plays a major role in apoptosis of neurons [Kuida et al., 1996]. Translocation of cytochrome *c* from intermembrane space of mitochondria to cytoplasm is a crucial step in apoptosis [Liu et al., 1996; Kluck et al., 1997; Yang et al., 1997; Zou et al., 1997]. Cytochrome *c* released from the mitochondria forms a complex with Apaf-1, and activates caspase-9, resulting in caspase-3 activation.

Abbreviations used: Caspase, aspartate-specific cysteine protease; D-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; D-MCA, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; DMSO, dimethyl sulfoxide; IGF-1, insulin-like growth factor-1; NO, nitric oxide; PAGE, polyacrylamide gel electrophoresis; PI3-kinase, phosphatidylinositol 3-kinase; SDS, sodium dodecyl sulfate; SIN-1, 3-morpholinosydnonimine; V-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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Nitric oxide (NO) is synthesized from L-arginine by NO synthase and mediates a number of physiological processes [McDonald and Murad, 1995]. In the central nervous system, physiological level of NO acts as an intercellular messenger [Dawson and Dawson, 1998]. However, NO has been shown to induce neuronal cell death when produced in excess [Iadecola, 1997]. Cytotoxicity is associated with highly reactive peroxynitrite formed by the reaction of NO with superoxide [Beckman et al., 1990]. Peroxynitrite is involved in a wide range of pathological processes including brain ischemia and neurodegeneration such as Alzheimer's disease and Parkinson's disease [Ischiropoulos, 1998], and is also reported to cause neuronal cell death [Bonfoco et al., 1995].

In the present study, we examined the role of IGF-1 on peroxynitrite-induced cell death and evaluated the relationship among cytochrome *c* release, caspase activation, and cell death in human neuroblastoma SH-SY5Y cells. We report here that IGF-1 inhibited caspase-3 activation and cell death but failed to prevent cytochrome *c* release induced by peroxynitrite. These findings suggest that IGF-1 protects peroxynitrite-induced cell death downstream of cytochrome *c* release.

MATERIALS AND METHODS

Materials

RPMI1640 medium, IGF-1, wortmannin, LY294002, and horse-heart cytochrome *c* were purchased from Sigma (St. Louis, MO). Benzyloxycarbonyl- Asp-Glu-Val-Asp-fluoromethylketone (D-FMK) was from Calbiochem (La Jolla, CA). Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (D-MCA), and benzyloxycarbonyl- Val-Ala-Asp-fluoromethylketone (V-FMK) were from Peptide Institute (Osaka, Japan). 3-morpholinosydnonimine (SIN-1) was from Dojindo Laboratories, Inc. (Kumamoto, Japan). Anti-cytochrome *c* antibody was from R&D (Minneapolis, MN). Anti-phospho-Akt and anti-cleaved caspase-3 (D175) antibodies were from New England Biolabs (Beverly, CA). Anti-Bcl-2 antibody was from DAKO (Copenhagen, Denmark). Other chemicals used were commercially available and of analytical grade.

Cell Culture and Treatment

SH-SY5Y cell line was a gift from Dr. Wolfgang Sadee (University of California, San Francisco). The cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum containing 100 μg/ml streptomycin, 100 IU/ml penicillin, and 1 μl/ml amphotericin B. Cells were plated on dishes at 5×10^4 cells/cm^2 and incubated for 24 h. The medium was changed to serum-free medium 30 min before the treatments with various reagents. Wortmannin, LY294002, and PD98059 were dissolved in dimethyl sulfoxide (DMSO) and added to the media (the solvent was finally diluted to 0.2%). When reagents dissolved in DMSO were used, equal volume of the solvent was added to the control media. Cell death was evaluated using 0.4% trypan blue exclusion test. The percentage of cell death was expressed as the percentage of stained cells as a fraction of the total number of cells. Approximately 1,000 cells were counted per group.

Analysis of DNA Fragmentation

DNA was isolated and purified using Apoptosis Ladder Detection Kit (Wako, Osaka, Japan) and subjected to electrophoresis in 1.5% agarose gels. DNA was visualized by ethidium bromide.

Preparation of Cytosolic Fractions

Cytosolic fractions were prepared as described [Liu et al., 1996]. Briefly, cells were suspended in 20 mM HEPES buffer (pH 7.4) containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin, and disrupted with a Dounce homogenizer. The supernatant at 100,000*g* for 1 h was used as cytosolic fractions.

Immunoblotting

To prepare total cell lysate, cells were lysed in 62.5 mM Tris-HCl (pH 6.8) buffer containing 2% sodium dodecyl sulfate (SDS), 1% glycerol, 50 mM dithiothreitol, and 0.1% bromphenol blue. For analysis of cytochrome *c* release, cytosolic fraction was prepared as described above. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to polyvinylidene difluoride membranes (Millipore, Bedford). The membranes were blocked with 20 mM Tris buffer (pH 7.6) containing 137 mM NaCl, 5% dry milk and 0.1% Tween-20 (TBST) for 1 h at room temperature, and probed with antibodies to cytochrome *c* (1:2,000), phospho-Akt (1:2,000),

cleaved caspase-3 (1:1,000), or Bcl-2 (1:500) overnight at 4°C in TBST. After washing three times with TBST, the membranes were probed with a horseradish peroxidase-linked antibody for 1 h at room temperature, and visualized by an enhanced chemiluminescence system (Amersham, Arlington Heights, IL, USA). In some experiments, the membranes were stripped and reprobed with other antibodies according to the manufacturer's instructions (Pierce, Rockford, IL).

Caspase-3-Like Activity Assay

Cells were lysed for 20 min in 20 mM Tris buffer (pH 7.4) containing 1% Triton X-100, 150 mM NaCl, 1 mM DTT, 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, and 2.5 μg/ml aprotinin. After the centrifugation at 10,000*g* for 20 min, supernatants were incubated with D-MCA for 60 min at 37°C. Caspase-3-like activities were determined by monitoring the increased fluorescence intensities (excitation at 380 nm and emission at 460 nm).

In Vitro Assay for Cytochrome *c*-Dependent Activation of Caspase

In vitro assay for cytochrome *c*-dependent activation of caspase-3 was essentially performed as described [Liu et al., 1996]. Cytosolic fraction (100 μg protein) was incubated with 10 μM horse-heart cytochrome *c* and 1 mM dATP at 37°C for 1 h in a total volume of 100 μl. After the incubation, the activation of caspase-3 was analysed by a fluorogenic method.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical differences between groups were determined using Tukey test. $P < 0.05$ was considered significant.

RESULTS

IGF-1 Protects Peroxynitrite-Induced Cell Death

We examined the effect of IGF-1 on peroxynitrite-induced cell death in SH-SY5Y cells. Cells were treated with IGF-1 (0.1-10 nM) for 24 h in the presence of 1 mM SIN-1. Cell death was assessed using a trypan blue dye exclusion test. Consistent with our previous report [Saeki et al., 2000], SIN-1 caused approximately 40% cell death. Treatment with IGF-1 prevented SIN-1-induced cell death in a concentration-dependent manner (Fig. 1). We also analysed DNA fragmentation of the cells treated with SIN-1 (1 mM) by agarose gel electrophoresis. SH-SY5Y cells showed DNA fragmentation by SIN-1 treatment (Fig. 2). Simultaneous addition of IGF-1 (10 nM) inhibited the DNA fragmentation.

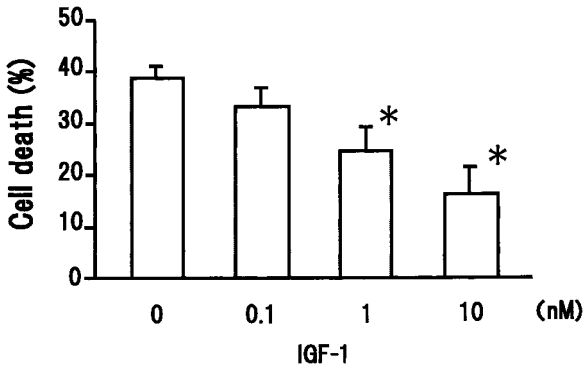

Fig. 1. Protection of SIN-1-induced cell death by IGF-1. Indicated concentrations (0.1–10 nM) of IGF-1 were added to the medium 30 min after the treatment of SIN-1 (1 mM). Cell viability was calculated by the failure to exclude trypan blue 24 h after the SIN-1 treatment. Data are given as means ± SEM from three experiments. *, $P < 0.01$ compared with that of treatment with SIN-1 alone (0).

Protection of Cell Death by IGF-1 is PI3-Kinase-Dependent

To determine whether the effect of IGF-1 involves the PI3-kinase pathway, we tested the effect of wortmannin, an inhibitor of PI3-kinase, on the protection of cell death by IGF-1. Simultaneous treatment of SH-SY5Y cells with IGF-1 (10 nM) and wortmannin (100 nM) inhibited the protective effect of IGF-1 on SIN-1-induced cell death (Fig. 3A). Wortmannin alone had neither effect on the survival of SH-SY5Y cells nor SIN-1-induced cell death. Similar results were obtained with another inhibitor of PI3-kinase, LY294002 (Fig. 3B). Akt, a serine/threonine kinase, is a downstream effector of PI3-kinase, and phosphorylated by PI3-kinase. Thus, we assessed the extent of phosphorylation of Akt on Ser-473, which is thought to reflect Akt activation. SH-SY5Y cells were treated with 10 nM IGF-1 for 15 min and the phosphorylation of Akt was evaluated using specific antibody that recognizes Akt phosphorylated at Ser-473. Treatment with 10 nM IGF-1 induced the phosphorylation of Akt, which was abolished by 100 nM wortmannin (Fig. 4A) or 10 nM LY294002 (Fig. 4B).

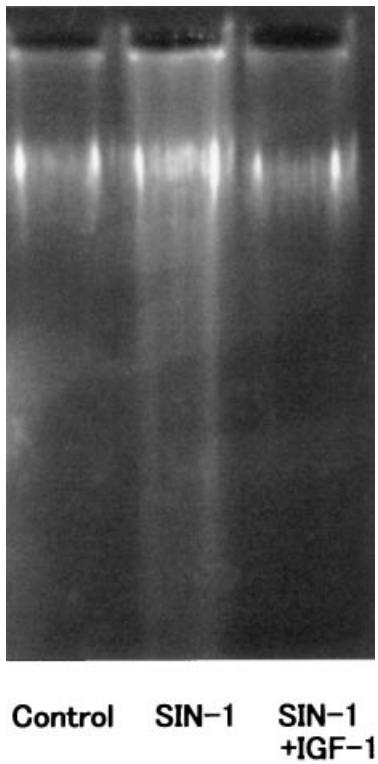

Fig. 2. Agarose gel electrophoresis of DNA fragmentation. Twenty-four hours after SIN-1 (1 mM) treatment, cells were collected. IGF-1 (10 nM) was added to the medium 30 min after the addition of SIN-1. **Lane 1**, control; **Lane 2**, 1 mM SIN-1; **Lane 3**, 1mM SIN-1 plus 10 nM IGF-1. Control indicates cells treated with vehicle.

IGF-1 Inhibits Caspase-3-Like Protease Activation Induced by SIN-1

The caspase family has been suggested to play an important role in neuronal cell death. We investigated whether caspase-3 is involved in peroxynitrite-induced cell death. Exposure of the cells to 1 mM SIN-1 increased caspase-3-like protease activity in a time-dependent manner (Fig. 5A). Maximal activity was detected at 24 h after the SIN-1 treatment. D-FMK (100 μM), an inhibitor of caspase-3, completely inhibited the increase in caspase-3-like protease activity induced by SIN-1 (Fig. 5B). We also examined effects of IGF-1 on SIN-1-induced activation of caspase-3. IGF-1 partially prevented the increase in caspase-3-like protease activity induced by SIN-1, and the effect of IGF-1 was completely inhibited by 100 nM wortmannin (Fig. 5B). During the activation of procaspase-3 with proteolysis, two fragments of p20 and p17 are produced. To confirm the activation of caspase-3, we used specific antibody against the fragments for immunoblotting analysis. The

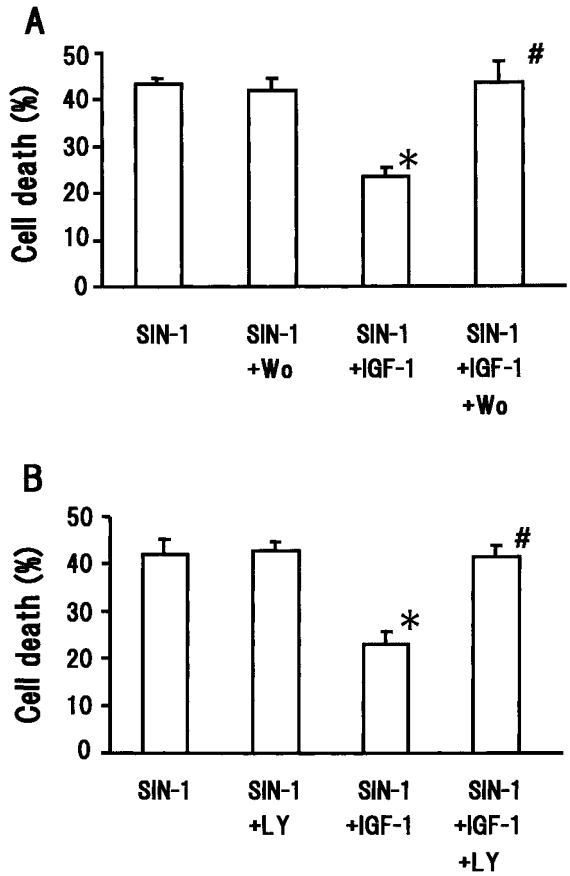

Fig. 3. Blockade of protective effect of IGF-1 by PI3-kinase inhibitors. IGF-1 (10 nM) and wortmannin (**A**, Wo, 100 nM) or LY294002 (**B**, LY, 10 nM) were added to the medium 30 min after the treatment of SIN-1 (1 mM). Cell viability was calculated 24 h after the SIN-1 treatment. Data are given as means ± SEM from three experiments from three experiments. *, $P < 0.01$ compared with that of treatment with SIN-1 alone. #, $P < 0.01$ compared with that of treatment with SIN-1 plus IGF-1.

p20 and p17 bands were present in the lysate of SH-SY5Y cells treated with SIN-1, and were reduced when treated with IGF-1 (Fig. 5C). To address whether caspase-3 contributes to SIN-1-induced cell death, we examined the effect of D-FMK on SIN-1-induced cell death. D-FMK (100 μM) partially prevented SIN-1-induced cell death (Fig. 6). Moreover, V-FMK (50 μM), a non-specific-caspase inhibitor, completely prevented SIN-1-induced cell death.

IGF-1 Does Not Reduce Cytochrome *c* Release Induced by SIN-1

During apoptosis, an important pathway leading to the activation of caspases is the release of cytochrome *c* from the mitochondria. Cytochrome *c* release was analysed in the cytosolic fraction obtained from SH-SY5Y cells 24 h after SIN-1 (1 mM) treatment. Figure 7A

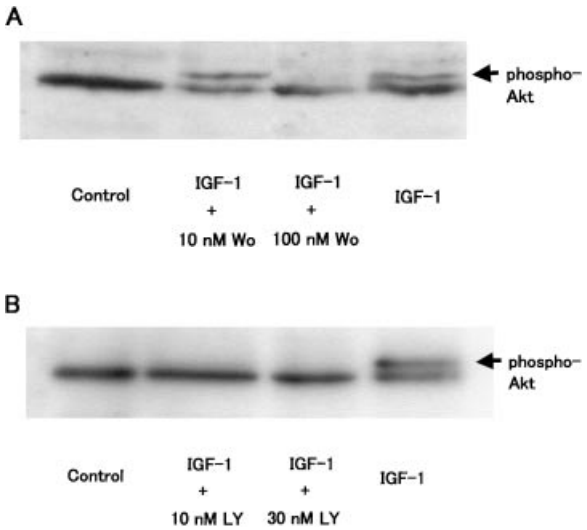

Fig. 4. Phosphorylation of Akt by IGF-1. Thirty minutes after pre-incubation with wortmannin (**A**, Wo, 10, 100 nM) or LY294002 (**B**, LY, 10, 30 nM), cells were treated with IGF-1 (10 nM) for 15 min. Control indicates cells treated with vehicle. Total cell lysates were subjected to SDS–PAGE and immunoblotted with antibody that recognizes serine-473 phosphorylated Akt. A given immunoblot is a representative of three independent experiments.

shows that SIN-1 increased the amount of cytochrome *c* in the cytosolic fraction. D-FMK (100 μM) was ineffective in preventing the release of cytochrome *c*, indicating that cytochrome *c* release is upstream of caspase-3 activation. As shown in Figure 7B, IGF-1 had no effect on the release of cytochrome *c* in SIN-1 treated SH-SY5Y cells. Since cytochrome *c* oxidase subunit II, an inner mitochondrial membrane protein, was not detected in the cytosolic fractions, there was no contamination of intact mitochondria in the prepared cytosolic fraction (data not shown).

IGF-1 Inhibits Cytochrome *c*-Induced Activation of Caspase-3

To confirm whether IGF-I inhibits cytochrome *c*-induced activation of caspase-3, we performed in vitro experiments using cytosolic fractions prepared from IGF-1-treated or non-treated cells. As reported previously [Liu et al., 1996], addition of cytochrome *c* and dATP to the cytosolic fractions obtained from non-treated cells resulted in caspase-3-like activation (Fig. 8A). However, caspase-3-like activation was reduced in IGF-1-treated cytosolic fractions. We also analysed the proteolytic processing of pro-caspase-3 by immunoblotting. By the addition of cytochrome *c* and dATP, the p20 and

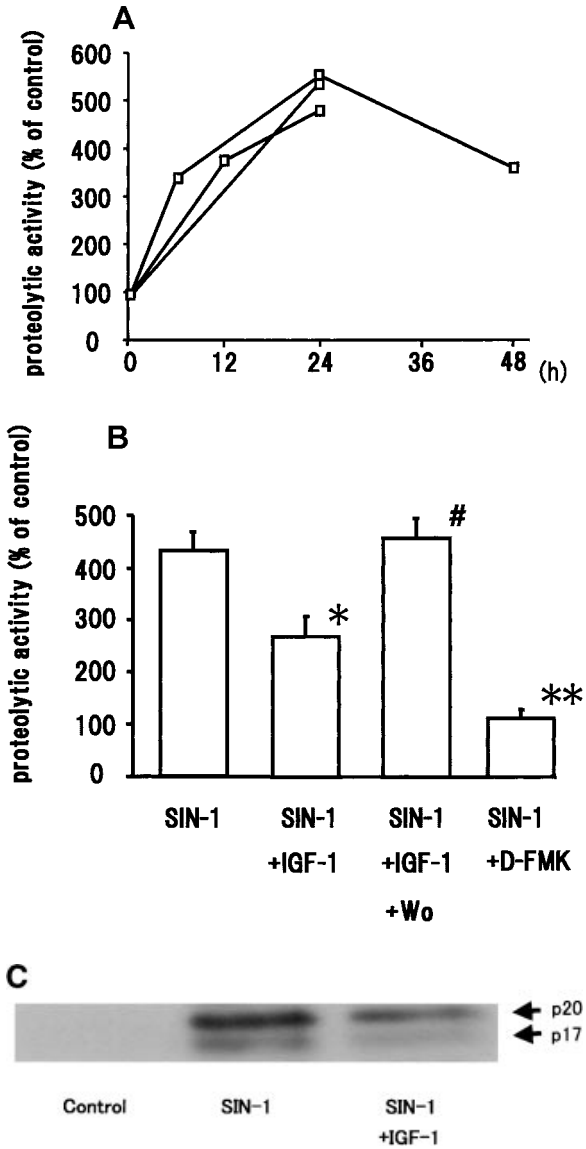

Fig. 5. Inhibition of SIN-1-induced caspase-3 activation by IGF-1. **A**: At the indicated time after SIN-1 (1 mM) treatment, cells were collected. Cell lysate (25 μg protein) was incubated with D-MCA for 60 min at 37°C. Caspase-3-like protease activity was determined by cleavage of fluorogenic substrate. The proteolytic activity at time 0 is defined as 100%. Data from three different experiments are presented. **B**: IGF-1 (10 nM) with or without wortmannin (Wo, 100 nM) were added to the medium 30 min after the treatment of SIN-1 (1 mM). D-FMK (100 μM) was added to the medium 30 min before the SIN-1 treatment. Caspase-3-like protease activity was determined by cleavage of fluorogenic substrate 24 h after the SIN-1 treatment. The proteolytic activity of a lysate from cells treated with vehicle is defined as 100%. *, $P < 0.05$; **, $P < 0.01$ compared with that of treatment with SIN-1. #, $P < 0.05$ compared with that of treatment with SIN-1 plus IGF-1. **C**: Total cell lysates 24 h after the SIN-1 addition were subjected to immunoblotting with antibody to cleaved products of procaspase-3. Control indicates cells treated with vehicle. A given immunoblot is a representative of three independent experiments. Arrows indicate the positions of cleaved products of procaspase-3 (p20 and p17).

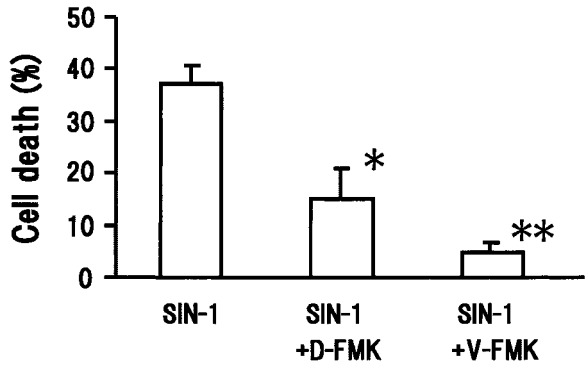

Fig. 6. Protection of SIN-1-induced cell death by caspase inhibitors. V-FMK (50 μM) or D-FMK (100 μM) was added to the medium 30 min before the treatment of SIN-1 (1 mM). Cell viability was calculated 24 h after the SIN-1 treatment. Data are given as means ± SEM from three experiments. *, $P < 0.05$; **, $P < 0.01$ compared with that of treatment with SIN-1.

p17 bands appeared in the cytosolic fractions obtained from non-treated cells, and were reduced in the fractions from cells treated with IGF-1 (Fig. 8B). Taken together, these findings demonstrate that IGF-1 inhibits cytochrome *c*-induced activation of caspase-3 downstream of cytochrome *c* release.

IGF-1 Does not Affect the Expression Levels of Bcl-2

One possible mechanism by which IGF-1 protects SIN-1-induced cell death is to increase the amount of endogenous Bcl-2. We conducted immunoblot analysis of cells treated with or without IGF-1 using anti-Bcl-2 antibody. There was no difference between the protein amounts of Bcl-2 in IGF-1 treated and non-treated cells (Fig. 9).

DISCUSSION

In this study, we showed that IGF-1 prevented peroxynitrite-induced cell death. IGF-1 is a well-established neuronal mitogen. A key role of IGF-1 in brain development has been shown in vivo using knockout mice [D'Ercole et al., 1996]. In addition to its mitogenic effect, recent evidence suggests that IGF-1 is also a survival factor for neurons. Serum withdrawal induces apoptosis in many types of cultured neuronal cells. For example, several neuroblastoma cell lines such as NG108 cells and NB 2a cells

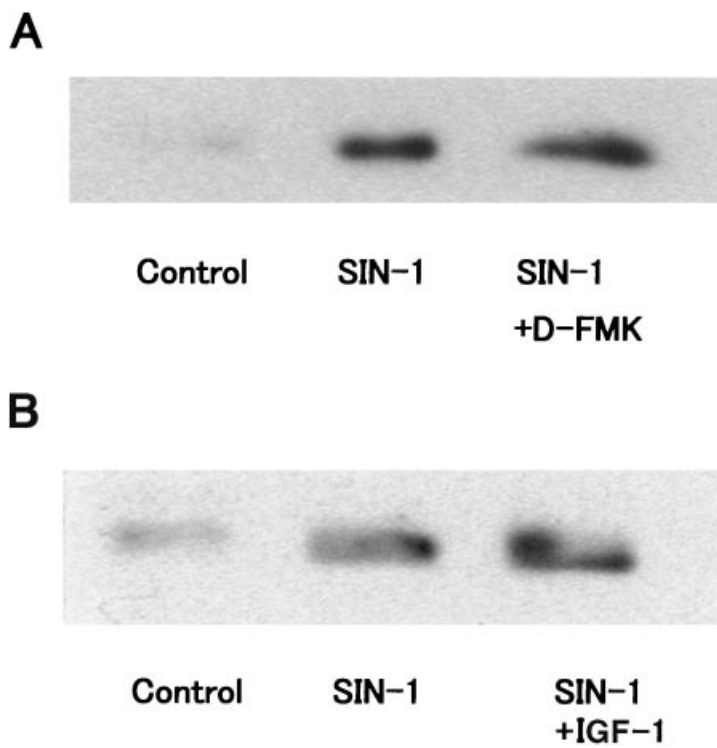

Fig. 7. Cytochrome *c* release induced by SIN-1. D-FMK (**A**, 100 μM) was added to the medium 30 min before the treatment of SIN-1 (1 mM). IGF-1 (**B**, 10 nM) was added to the medium 30 min after the treatment of SIN-1. Cytosolic fraction 24 h after the SIN-1 addition was subjected to immunoblotting with antibody to cytochrome *c*. Control indicates cells treated with vehicle. A given immunoblot is a representative of three independent experiments.

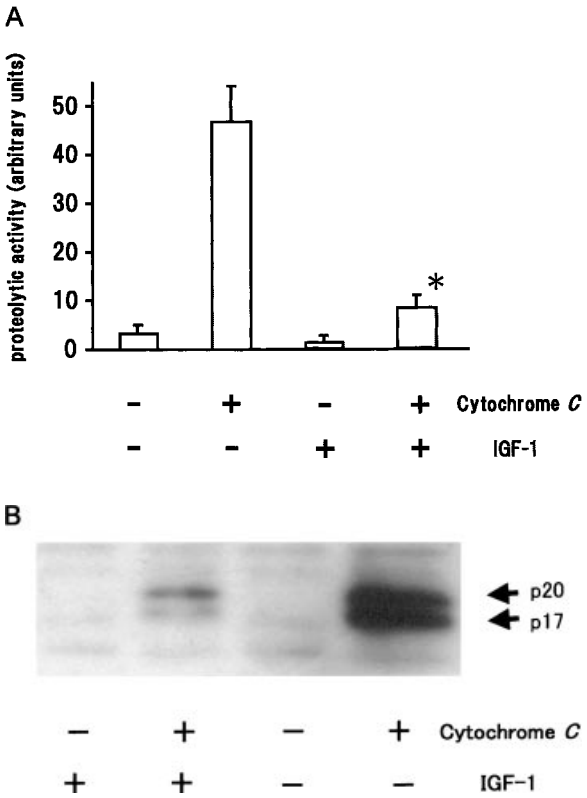

Fig. 8. In vitro activation of caspase-3 by cytochrome *c*. After the incubation with or without 10 μM cytochrome *c* for 60 min at 37 °C, cytosolic fractions (100 μg protein) prepared from cells treated with IGF-1 or without IGF-1 were analysed by fluorogenic method (**A**) or immunoblotting with antibody to cleaved products of procaspase-3 (**B**). A: The proteolytic activity of IGF-1-treated cells (no addition of cytochrome *c*) is defined as 1 arbitrary unit. Data are given as means ± SEM from three experiments. *, $P < 0.01$ compared with that of cytochrome *c* added-fraction from IGF-1-non-treated cells. B: A given immunoblot is a representative of three independent experiments. Arrows indicate the positions of cleaved products of procaspase-3 (p20 and p17).

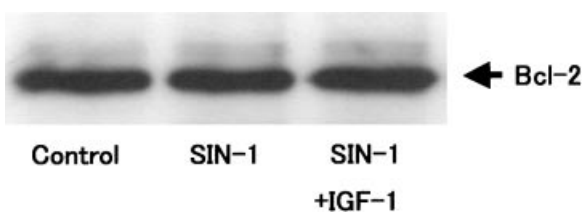

Fig. 9. Effect of IGF-1 on the expression of Bcl-2. IGF-1 (10 nM) was added to the medium 30 min after the addition of SIN-1 (1 mM). Total cell lysates 24 h after the SIN-1 addition were subjected to immunoblotting with antibody to Bcl-2. Control indicates cells treated with vehicle. A given immunoblot is a representative of three independent experiments.

undergo apoptosis when switched to serum-free medium [Solovyan et al., 1998; Yano et al., 1998]. In this study, however, serum-free conditions did not induce death of SH-SY5Y cells. This is consistent with previous report [Matthews and Feldman, 1996].

PI3-kinase has been shown to be implicated in the signaling of IGF-1-mediated cell survival. PI3-kinase is a heterodimer of a regulatory subunit and a catalytic subunit [Carpenter et al., 1990]. Wortmannin is a fungal toxin that covalently binds to and blocks the activity of the catalytic subunit [Arcaro and Wymann, 1993; Kimura et al., 1994]. Consistent with findings from the experiments using other cells [Yao and Cooper, 1995; Parrizas et al., 1997], wortmannin blocked the ability of IGF-1 to protect SH-SY5Y cells against peroxynitrite-induced cell death. To confirm our finding that PI3-kinase is involved in IGF-1-mediated cell survival, we used another specific inhibitor of PI3-kinase, LY294002. LY294002 is a synthetic bioflavonoid that reversibly binds to and inhibits the catalytic subunit [Vlahos et al., 1994]. LY294002 showed similar blocking effects on IGF-1-evoked inhibition of cell death, indicating that IGF-1 prevents peroxynitrite-induced cell death via PI3-kinase activation.

The effect of PI3-kinase is transmitted through its production of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, which results in activation of Akt. We assessed the extent of phosphorylation of Akt on serine-473, which is thought to reflect Akt activation [Alessi et al., 1996]. The phosphorylation of Akt was inhibited by wortmannin or LY294002. Taken together, these results suggest that IGF-1 protects peroxynitrite-induced cell death via the activation of PI3-kinase/Akt pathway.

In neurons, there is considerable evidence that caspase-3 plays an important role for cell death in response to ischemia and excitotoxicity [Namura et al., 1998; Tenneti and Lipton, 2000]. It has been reported that NO induces neuronal apoptosis via caspase-3-like activation [Tamatani et al., 1998b]. Peroxynitrite-induced cell death may be a mixture of apoptosis and necrosis. In fact, DNA ladder pattern was not clear when DNA was isolated from SIN-1-treated cells. When DNA was isolated from H_2O_2-treated cells, ladder pattern was clear. A broad-spectrum caspase inhibitor (50 μM) completely prevented SIN-1-induced cell death whereas

a specific caspase-3 inhibitor, used at a 2-fold higher dose, only partially prevented cell death, suggesting that in addition to caspase-3, other caspases may also be involved in peroxynitrite-induced cell death.

It has been shown that the release of cytochrome *c* from the mitochondria results in the formation of Apaf-1 and caspase-9, and subsequently activates caspase-3 [Kluck et al., 1997; Yang et al., 1997; Zou et al., 1997]. The exact mechanism how cytochrome *c* is released during peroxynitrite-induced cell death remains unknown. The Bcl-2 family member Bax has been reported to regulate cytochrome *c* release [Hsu et al., 1997]. It has also been reported that Bax mediates NO-induced cell death in SH-SY5Y cells [Ghatan et al., 2000]. In this study, IGF-1 prevented caspase activation, but failed to prevent the release of cytochrome *c* induced by peroxynitrite, suggesting that IGF-1 inhibits cell death downstream of cytochrome *c* release. Similar findings are reported in cerebellar neurons or motor neurons where IGF-1 protected cell death without inhibiting cytochrome *c* release [Gleichmann et al., 2000; Zhou et al., 2000].

Recent studies suggest a possible link between the effects of IGF-1 and Bcl-2 on cell death. For example, in a neuronal hyperosmotic stress model, cell death was blocked by IGF-1 receptor activation, and this effect was associated with the increased expression of Bcl-2 [Singleton et al., 1996; Golen et al., 2000]. Matsuzaki et al. [1999] also reported that IGF-1 inhibits NO-induced apoptosis by modulating NO-induced changes in Bcl-2 expression. The primary site of action of Bcl-2 appears to be in the mitochondria because its overexpression inhibits the release of cytochrome *c* [Kluck et al., 1997; Yang et al., 1997]. However, we observed that IGF-1 did not affect the expression levels of Bcl-2. This result suggests that IGF-1 may prevent peroxynitrite-induced cell death through a mechanism that is independent of Bcl-2 induction.

Recent studies suggest that in certain cell types including neurons, there may be a post-mitochondrial mechanism to inhibit cell death. For example, it has been reported that Hsp70 inhibits apoptosis downstream of cytochrome *c* release without preventing cytochrome *c* redistribution [Xanthoudakis and Nicholson, 2000]. Inhibitors of apoptosis proteins (IAPs), identified in the genome of baculovirus, are also reported to regulate the cytochrome *c*-mediated caspase activation. IAPs suppress apoptosis by preventing the proteolytic processing of procaspase and inhibiting the enzymatic activity of mature caspases [Deveraux and Reed, 1999]. Several mammalian IAPs including XIAP, c-IAP1, c-IAP2, and survivin have been identified. In addition, it has been reported that the ability of microinjected cytochrome *c* to trigger apoptosis depends on other signals induced by nerve growth factor (NGF) [Deshmukh and Johnson, 1998]. Although the molecular basis is not clear, it is hypothesized that growth factors including IGF-1 may induce or activate anti-apoptotic proteins such as IAPs and inhibit caspase activation in SH-SY5Y cells.

In conclusion, our data indicate that IGF-1 inhibits peroxynitrite-induced cell death downstream of cytochrome *c* release. The ability of IGF-1 to regulate cell death may provide a potential molecular mechanism preventing neuronal cells death induced by an accidental leakage of cytochrome *c* from the mitochondria.

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