

Effects of insulin-like growth factor-1 on okadaic acid-induced apoptosis in SH-SY5Y cells

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Abstract

The effects of insulin-like growth factor-1 (IGF-1) on the cytotoxicity and apoptosis induced by okadaic acid (OA) in SH-SY5Y cells were investigated. Cell viability was measured using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. Early and late apoptosis/necrosis were analyzed by flow cytometry using Annexin V and propidium iodide (PI) double-staining. Caspase-3 activation was detected by Western blot analysis. Preincubation with IGF-1 for 24 h prevented cytotoxicity induced by 40 nM OA given for 24 h, and the MTT value significantly increased. Incubation with 20 nM OA for 24 h caused a marked increase in the percentage of early apoptotic and late apoptotic/necrotic cells, which was not dependent on the activation of caspase-3. OA-induced apoptosis was significantly decreased by pretreatment with 10 ng/ml of IGF-1 for 24 h. The results supported the hypothesis that IGF-1 may be useful in the treatment of Alzheimer's disease.

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1. Introduction

Intracellular neurofibrillary tangles (NFT) (Kowall et al., 1991) are a hallmark of neuropathological lesions in Alzheimer's disease (AD). The main component of NFT is paired helical filaments (PHFs), which are made up of microtubule-associated protein tau in a hyper/abnormal phosphorylated form (Lee et al., 1991; Matsuo et al., 1994; Spillantini and Goedert, 1998). Hyperphosphorylation of tau dissociate tau from microtubules, disrupting the neuronal cytoskeleton and interfering with cellular transport mechanisms (Vincent et al., 1994). Although the mechanism remains unclear,

decreased protein phosphatase (PP) activity, especially of PP2A and PP1, is believed to be a cause of the abnormal hyperphosphorylation of tau (Matsuo et al., 1994; Gong et al., 1995). OA is a potent specific inhibitor of serine/threonine PPI and PP2A (Bialojan and Takai, 1988). It induces hyperphosphorylation of tau and neuronal death similar to those found in AD (Arias et al., 1993; Dupont-Wallois et al., 1995; Arendt et al., 1998; Kim et al., 1999), and can be considered as a research model of AD. When OA is administered to rats, PHFs in the hippocampus have been detected (Lee et al., 2000). SH-SY5Y is a thrice-cloned subline of the neuroblastoma cell line SK-N-SH which was established in 1970. Treatment of SH-SY5Y human neuroblastoma cells with OA can induce changes similar to those occurring in vivo in AD.

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IGF-1 is a pleiotropic factor with structural and functional homologies to IGF-2 and insulin. IGF-1 and its specific receptor are distributed in various neuroanatomical regions, especially concentrated in the hippocampus (Kar et al., 1993). Insulin and IGF-1 have important functions in the brain, including metabolic, neurotrophic, neuromodulatory and neuroendocrine actions (Torres-Aleman, 2000). Recently, IGF-1 has gained increasing attention in relation to the pathogenesis of AD (Gasparini et al., 2002). Insulin and IGF-1 levels in AD patients are altered and the levels of IGF-1 binding sites are significantly increased in cortical areas of AD brains (Crews et al., 1992). IGF-1 seems to protect hippocampal neurons from the toxicity of amyloid β (A β) protein (Dore et al., 1997). Additionally, IGF-1 may be a key factor in regulating the clearance of A β from the brain through carrier-mediated transport (Carro et al., 2002).

We have explored in this work the neuroprotective and anti-apoptotic effects of IGF-1 on OA-induced cytotoxicity. IGF-1 prevented cytotoxicity and apoptosis caused by the phosphatase inhibitor, OA, in SH-SY5Y cells.

2. Materials and methods

2.1. Reagents

Media, 0.25% trypsin–EDTA, and antibiotics for cell culture were from Invitrogen. Fetal bovine serum (FBS) and L-glutamine were from Hyclone. OA (Sigma) was dissolved in dimethyl sulfoxide (DMSO) at 100 μ M as a stock solution. Recombinant human IGF-1 was purchased from PeproTech Inc., and dissolved in sterile ddH₂O at 100 μ g/ml as a stock solution. This stock was aliquoted and stored at -20°C . Rabbit anti-caspase-3 primary antibody was purchased from Santa Cruz.

2.2. SH-SY5Y cells culture and experiment treatments

Differentiated human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. In the experiments, cells were replated at low density (20,000 cells/cm²), and cultured for 1 day prior to experimentation.

To determine the effects of IGF-1 on OA-induced cell death and apoptosis, SH-SY5Y cells were pretreated with IGF-1 at 1, 10, and 100 ng/ml, or vehicle, for 24 h prior to exposure to 20 or 40 nM OA for 24 h.

2.3. MTT assay

Ten microliters of MTT (5 mg/ml stock in PBS) was added to each well (96-well plate, 100 μ l medium/well), and incubated for 4 h. The insoluble blue formazan was solubilized with 100 μ l of DMSO, and OD values of the mixture were measured at 550 nm and 650 nm with a Bio-Rad microplate reader. All MTT assays involved not less than 4 separate samples, which were measured in triplicate. Survival of vehicle-treated control cells not exposed to OA was taken as 100%, with values for the other groups being given as a percentage of control.

2.4. Flow cytometric analysis using Annexin V–FITC and PI

To detect early apoptosis and late apoptosis/necrosis, cells were stained with FITC-conjugated Annexin V and PI. Approximately 1×10^6 cells were washed with cold PBS before being resuspended in 200 μ l cold $1 \times$ binding buffer. Ten microliters of Annexin V–FITC and 5 μ l PI were added and incubated for 15 min at room temperature in the dark. A further 300 μ l binding buffer was added to terminate the reaction, and flow cytometric analysis was made immediately of at least 10,000 cells with a FACSCalibur instrument (Becton Dickinson Immunocytometry System). The experiment was repeated 3 times and the results were averaged.

2.5. Western blotting analysis

Caspase-3 activation was determined by Western blot. Cells were lysed in complete lysis buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% SDS, 2 mM dithiothreitol (DTT), 10% glycerol and complete mini protease inhibitors (1 tablet/10 ml buffer; Roche). Insoluble materials were removed by centrifugation. The protein concentrations of the lysates were determined by the colorimetric bicinchoninic acid analysis (Pierce Chemical Company). Equal amount of protein was separated in a 10% SDS-PAGE (30 μ g/lane) for ~ 1.5 h at 120 V, and transferred on to nitrocellulose membranes at constant voltage (100 V) for 1.5 h. The membranes were blocked with 3% non-fat dry milk in TTBS (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20), and probed with rabbit anti-caspase-3 primary antibody at 4°C overnight. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, followed by enhanced chemiluminescence detection (Roche).

2.6. Statistical analysis

Data have been expressed as mean \pm SD (or SEM). Statistical significance was determined using one-way

ANOVA (SPSS). Differences were considered significant if a P value was <0.05 .

3. Results

3.1. Neuroprotective effects of IGF-1 against OA-induced cytotoxicity

Differentiated SH-SY5Y cells have elongated or polygonal shapes and long neurites (Fig. 1A). After incubation with 40 nM OA for 24 h, the morphology of the SH-SY5Y cells became round and the neurites disappeared (Fig. 1B). Cell viability was reduced to $\sim 58\%$. IGF-1 protected SH-SY5Y cells against OA-induced toxicity. Pretreatment with IGF-1 at 1, 10, and 100 ng/ml for 24 h prior to exposure to 40 nM OA for 24 h gave viabilities of 58%, 69%, and 72%, respectively (Fig. 1C). When treated with IGF-1 alone, the survival of neurons was not significantly affected.

3.2. Effects of IGF-1 on earlier apoptosis and late apoptosis/necrosis induced by OA

To observe the effects of IGF-1 on premature apoptosis and late apoptosis/necrosis induced by OA, SH-SY5Y cells were analyzed by flow cytometry, which readily distinguishes the former from the latter. In the premature stages of apoptosis, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet. Annexin V has a high affinity for PS, which identifies apoptosis at this earlier stage. The Annexin V $-$ /PI $-$ population can be regarded as normal cells, while Annexin V $+$ /PI $-$ cells are taken as earlier apoptosis, and Annexin V $+$ /PI $+$ as late apoptosis/secondary necrosis (Koopman et al., 1994; Homburg et al., 1995; Vermes et al., 1995).

Significant differences were observed between the control and OA-treated cells. After incubation with 20 nM OA for 24 h, the percentage of both Annexin V $+$ /PI $-$ and Annexin V $+$ /PI $+$ cells increased significantly from 0.75% to 1.30% and from 2.46% to 6.05%,

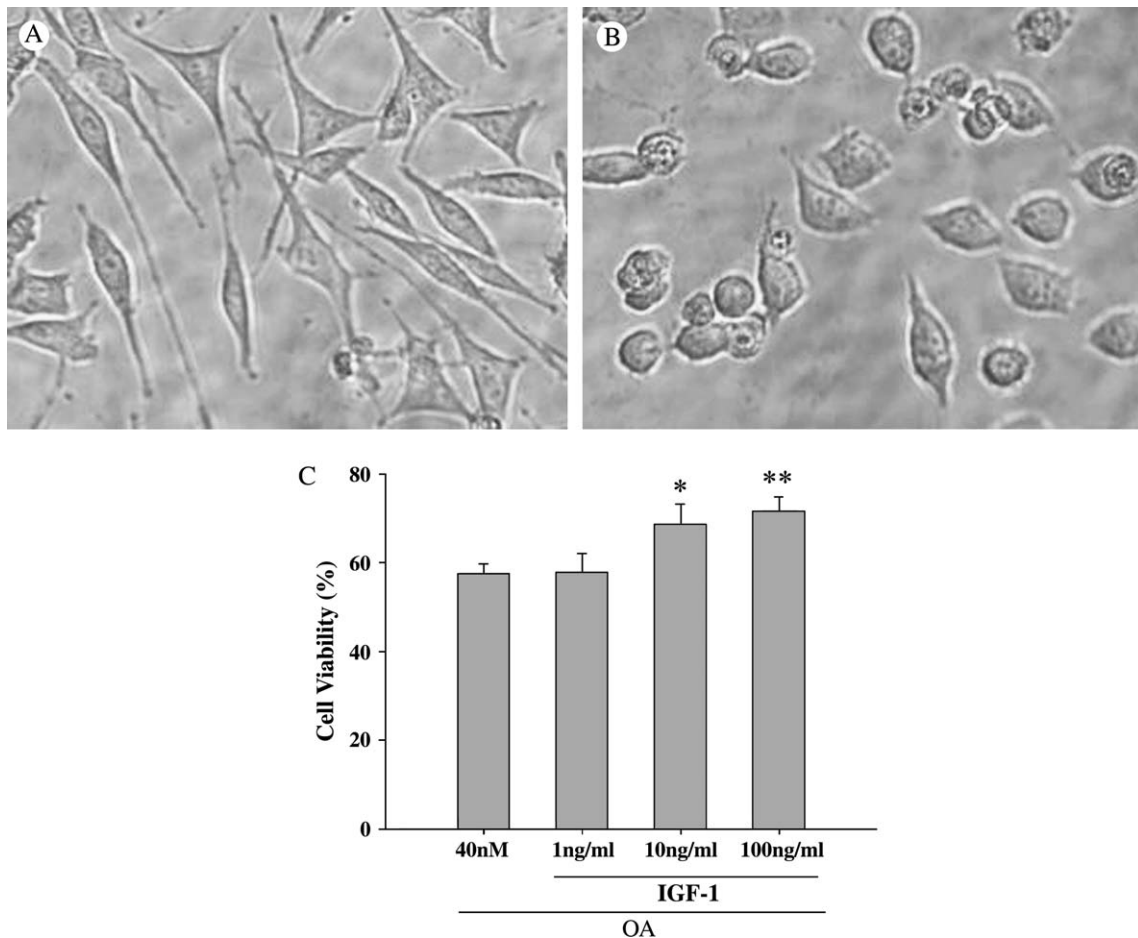


Fig. 1. Effects of IGF-1 on OA-induced cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were pretreated with different concentrations of IGF-1 for 24 h prior to exposure to 40 nM OA for 24 h. (A, B) The morphology of the normal (A) and the OA-treated SH-SY5Y cells (B) ($\times 400$). (C) Bar graph is presented as the percentage of control MTT value. Data are expressed as mean \pm SEM. *, $P < 0.05$; and **, $P < 0.01$ compared with OA-treated group.

respectively. Pretreatment with 10 ng/ml IGF-1 for 24 h prior to exposure to OA resulted in the values of Annexin V+/PI- and Annexin V+/PI+ cells significantly decreasing to 0.6% and 4.4%, respectively (see Fig. 2).

3.3. No caspase-3 activation involved in the cell apoptosis

To further investigate the relationship between IGF-1 protective effect on the OA-induced apoptosis and caspase-3 activity, SH-SY5Y cells were treated with OA in the presence or absence of IGF-1. Procaspase-3 expression level and caspase-3 activation were measured using immunoblot analysis with a caspase-3 specific antibody. OA treatment did not induce a significant reduction of procaspase-3 expression, and did not induce cleavage of caspase-3 (Fig. 3). This makes apoptosis induced by OA caspase-3-independent.

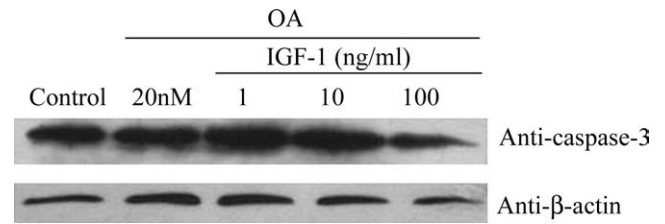


Fig. 3. Western blot analysis of caspase-3 activation in SH-SY5Y cells. SH-SY5Y cells were treated with different concentrations of IGF-1 for 24 h prior to exposure to 20 nM OA for 24 h. The blots showed the expression of procaspase-3 and no cleavage of caspase-3.

4. Discussion

The major findings of this study are 3-fold. First IGF-1 protected SH-SY5Y cells against OA-induced cytotoxicity. Second, IGF-1 decreased not only the percentage of late apoptotic/necrotic cells, but also early apoptotic cells. Third, these effects were caspase-3-independent.

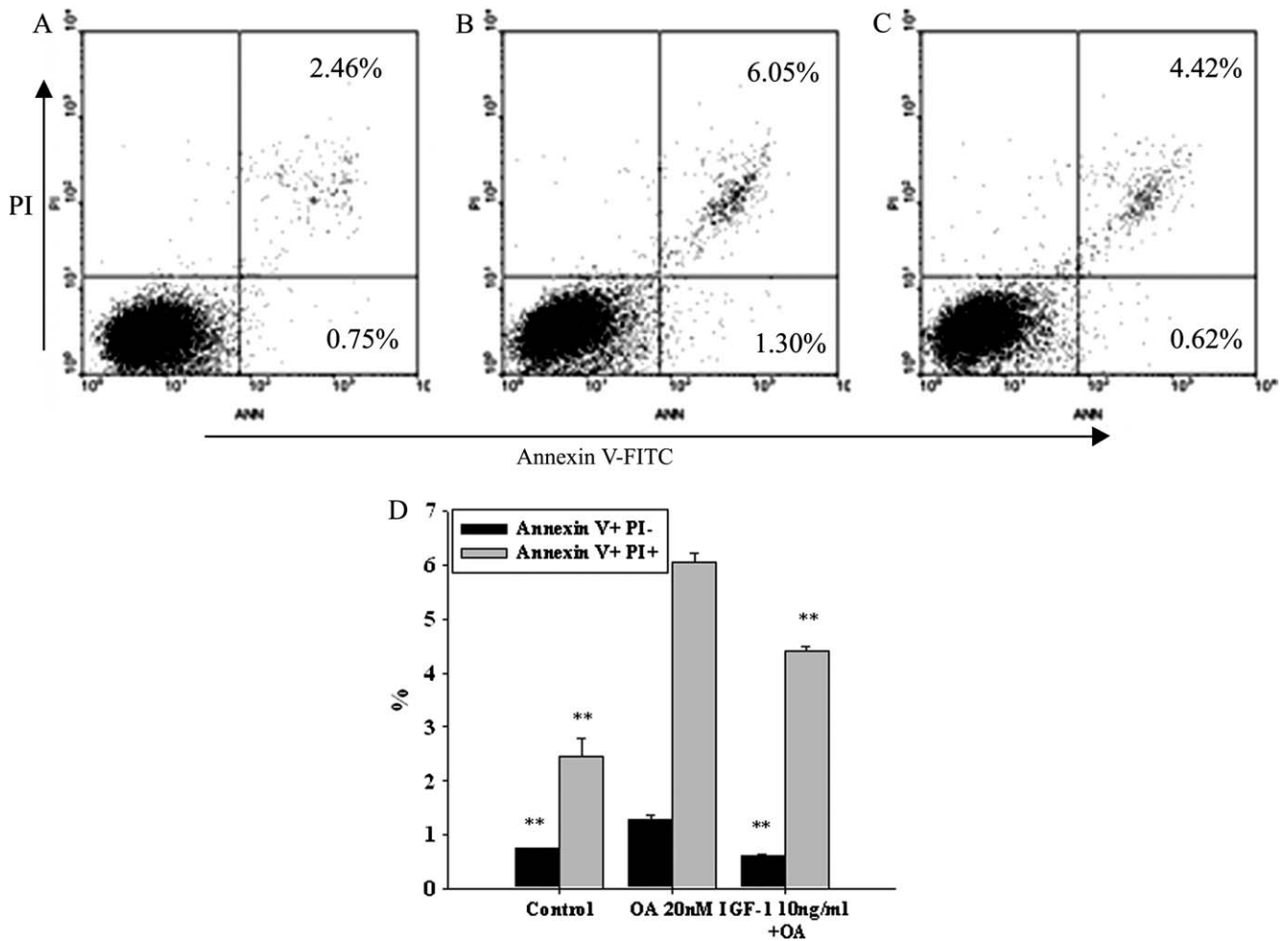


Fig. 2. Effects of IGF-1 on early apoptosis and late apoptosis/necrosis induced by OA in SH-SY5Y cells. (A) Control group; (B) OA-treated group; (C) SH-SY5Y cells were treated with 10 ng/ml IGF-1 for 24 h prior to exposure to 20 nM OA for 24 h; and (D) results were presented as percentage of gated cells. Data were expressed as mean ± SD. **, P < 0.01 compared with OA-treated group.

OA proved to be highly toxic to SH-SY5Y cells, inducing cytoplasmic shrinkage, rounding, and loss of neurites. IGF-1 protected SH-SY5Y cells against OA-induced toxicity, pretreatment with 100 ng/ml of IGF-1 for 24 h giving a viability of 24.5% compared with OA-treated group.

Apoptosis and necrosis are two modes of cell death in nucleated eukaryotic cells. Apoptosis is programmed cell death characterized by changes in condensation of nuclear chromatin, cytoplasmic blebbing, and exposure of PS residues on the outside of the plasma membrane (Cohen et al., 1992). Necrosis, on the other hand, is accidental cell death and shows the early phase features of mitochondrial swelling, rupture of the plasma membrane, and release of cytoplasmic constituents (Majno and Joris, 1995; Fiers et al., 1999). A key event during the early stage of apoptosis is that PS of the inner leaflet of the cell membrane appears in the outer leaflet, becoming a marker molecule for phagocytosis. Annexin V preferentially binds to PS, and can be used to detect expression of PS on the surface of apoptotic cells.

Although neuronal apoptosis is a normal event during development, many neurodegenerative diseases are thought to involve abnormal cell death that leads to damage of the nervous system. The toxicity of OA could also relate to their abilities to stimulate apoptotic genes/cellular events. In our study, OA not only decreased cell viability, but also increased the percentage of the apoptotic cells. There were significant differences between the control group and the OA-treated group. Additionally, IGF-1 has already been shown to block programmed cell death in various models (D'Mello et al., 1993; Sell et al., 1995). Hence, IGF-1 could act on necrosis and/or different stages of the apoptotic pathway to protect cells against OA-induced cell death.

Apoptosis involves the activation of a group of cysteine proteases or 'caspases' that are key factors that cleave their substrates at specific aspartate residues during apoptosis. More than 10 caspases have been identified and cloned. Caspases are synthesized as proproteins, possessing weak proteolytic activity. When cleaved at internal aspartate residues, procaspases are converted into mature enzymes with increased activity (Abraham and Shaham, 2004). Recently, the apoptotic effect of OA was found to involve caspase-3 activation (Li et al., 2001; Sandal et al., 2001); nevertheless, it was also shown that inhibition of caspase activity fails to prevent apoptosis in OA-treated cells, thereby suggesting that the PP inhibitor triggers activation of both caspase-dependent and caspase-independent death pathways (Sandal et al., 2001). We found that caspase-3 was not activated by OA in SH-SY5Y cells. Thus, the anti-apoptotic effect of IGF-1 seems unrelated to the inhibition of the activation of caspase-3.

New investigations suggest the existence of a caspase-independent pathway leading to cell death, its contri-

bution being dependent on the state of neuronal maturation (Johnson et al., 1999). Caspase-3 can be prominently activated in immature neurons, but less so in mature neurons (Hong et al., 2002). Recent studies also indicate that caspase activation does not always lead to cell death and, instead, might be important for cell differentiation (Abraham and Shaham, 2004). These reported findings are in line with the present observation that the cell death in differentiated SH-SY5Y cells is caspase-independent.

In summary, the phosphatase inhibitor, OA, induces apoptosis in SH-SY5Y cells via a mechanism that appears to not involve caspase-3 activation. Moreover, IGF-1 protects SH-SY5Y cells against OA-induced toxicity and apoptosis. These findings and other unique properties suggest that IGF-1 analogs could provide a novel and promising therapeutic strategy toward the treatment of various neurodegenerative diseases including AD.

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