Insulin-Like Growth Factor-I Prevents Apoptosis in Neurons after Nerve Growth Factor Withdrawal

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ABSTRACT: Insulin-like growth factor-I (IGF-I) is emerging as an important growth factor able to modulate the programmed cell death (PCD) pathway mediated by the cysteine-dependent aspartate proteases (caspases); however, little is known about the effect of IGF-I after nerve growth factor (NGF) withdrawal in neurons. To begin to understand the neuronal death-sparing effect of IGF-I under NGF-free conditions, we tested whether embryonic sensory dorsal root ganglion neurons (DRG) were able to survive in defined serum-free medium in the presence of IGF-I. We further studied the role of IGF-I signaling and caspase inhibition after NGF withdrawal. NGF withdrawal produced histological changes of apoptosis including chromatin condensation, shrinkage of the perikaryon and nucleus, retention of the plasma membrane, and deletion of single cells. Both IGF-I and Boc-aspartyl (OMe)-fluoromethylketone (BAF), a caspase inhibitor, equally reduced apoptosis after NGF withdrawal. The antiapoptotic effect of IGF-I was completely blocked by LY294002, an inhibitor of PI 3-kinase signaling, but not by the mitogen-activated protein (MAP) kinase/extracellular signal-regulated protein kinase (ERK) activated protein kinase inhibitor PD98059. Functional IGF-I receptors were extensively expressed both in rat and human DRG neurons, although they were most abundant in the neuronal growth cone. Collectively, these findings indicate that IGF-I, signaling though the PI-3 kinase pathway, is important in modulating PCD in cultured DRG neurons after NGF withdrawal, and IGF-I may be important in DRG embryogenesis. © 1998 John Wiley & Sons, Inc. J Neurobiol 36: 455–467, 1998

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Insulin-like growth factor-I (IGF-I) is a polypeptide essential for normal fetal, neonatal, and pubertal growth (Daughaday and Rotwein, 1989; Zackenfels et al., 1995). *In vitro,* IGF-I promotes the survival of cloned neural cell lines (Singleton et al., 1996a; Párrizas et al., 1997) and primary neurons (Zackenfels and Rohrer, 1993; Zackenfels et al., 1995; Miller et al., 1997; Sortino and Canonico, 1996). The biological actions of IGF-I are mediated by the type I IGF receptor (IGF-I receptor). Like IGF-I, there is developmental and tissue specific expression of the IGF-I receptor in the nervous system (Bondy et al., 1990; Lenoir and Honegger, 1983; van der Pal et al., 1988; Cohen et al., 1992).

In our laboratory, we are interested in the role

of IGF-I and the IGF-I receptor in the growth, survival, and differentiation of neurons. Our preliminary studies suggest that IGF-I promotes neuronal survival by inhibiting programmed cell death (PCD) (Matthews and Feldman, 1996; Singleton et al., 1996a,b; Matthews et al., 1997). IGF-I rescues SH-SY5Y human neuroblastoma cells and primary dorsal root ganglia (DRG) neurons from stress induced PCD (Matthews and Feldman, 1996; Russell and Feldman, 1996) via IGF-I:IGF-I receptor activation of the phosphatidyl-inositol (PI) 3-kinase pathway (Singleton et al., 1996a; Miller et al., 1997). Upon PI 3-kinase activation, IGF-I signaling increases the levels of the antiapoptotic bcl proteins (Singleton et al., 1996a) while blocking activation of the effectors of neural death, the cysteine-dependent aspartate proteases (caspases) (Singleton et al., 1996a).

In the current study, we asked whether IGF-I could rescue DRG neurons from PCD induced by nerve growth factor (NGF) withdrawal. We chose the experimental paradigm of NGF withdrawal in cultured DRG neurons because it provides a model for looking at the role of trophic factors during development. Neurons in the vertebrate nervous system, like DRG, become dependent on neurotrophic factors once they begin to innervate their targets (Scott and Davies, 1990). In embryonic DRG there is an increase in PCD from embryonic day 15 onward as target factors including NGF determine the number of surviving sensory neurons (Coggeshall et al., 1994). These sensory neurons are acutely sensitive to growth factor withdrawal and insufficient NGF results in PCD (Deckwerth and Johnson, 1993; Scott and Davies, 1990). We used the paradigm of NGF withdrawal to determine what role IGF-I may play in DRG embryogenesis.

We report that both rat and human DRG express the IGF-I receptor. *In vitro,* IGF-I maintains DRG neuronal survival after NGF withdrawal for at least 72 h. The antiapoptotic effect of IGF-I is mediated by PI 3-kinase and mimics the effect of caspase inhibitors such as Boc-aspartyl (OMe)-fluoromethylketone (BAF). Collectively, these results suggest a role for IGF-I and the IGF-I receptor in the development and maintenance of DRG neurons.

MATERIALS AND METHODS

DRG Culture

Dorsal root ganglion neurons from 15-day-old embryonic (E15) Sprague–Dawley rats (Harlan Sprague–Dawley,

Madison, WI) were aseptically plated on air-dried, collagen-coated plastic dishes as previously described (Windebank and Blexrud, 1986; Russell et al., 1994). Explants were incubated at 37°C with 5% carbon dioxide, in serum-free defined medium (SFDM) containing Eagle's minimal essential medium (EMEM; Gibco, Grand Island, NY) supplemented with 1.4 mM L-glutamine, 30 mM glucose, 30 nM selenium, 10 nM hydrocortisone, 10 nM β-estriadiol, and 100 μg/mL transferrin (Sigma Chemical Co., St. Louis, MO). We had previously observed that this medium produces equal or superior neuronal growth and survival between 0 and 96 h compared to medium containing calf serum. Control DRG were supplemented with 10 ng/mL 2.5S NGF (Bioproducts for Science, Madison, WI). We had previously obtained optimal DRG survival and neurite halo development in defined medium using this specific NGF product at this concentration (Russell and Feldman, 1996).

Dissociated DRG were prepared by incubating the whole DRG in 0.05% trypsin (Gibco) for 40 min at 37°C, centrifuging at 800 $\times$ g for 5 min, followed by trypsin removal. The cells were washed in EMEM, followed by gentle tituration through a Pasteur pipet with a flame-narrowed tip. To reduce the number of contaminating Schwann cells and fibroblasts, neurons were preplated on positively charged Primaria plates (Falcon, Franklin Lakes, NJ) for 45 min, which reduces the number of nonneuronal cells from 3% to 0.1% (Deckwerth and Johnson, 1994). The dissociated DRG neurons were plated at a density of approximately 5000 neurons/collagen-coated glass slide. To improve the quality of staining and counting, dissociated rat DRG cultures were used for all experiments except transmission electron microscopy (TEM). For TEM, whole DRG were used to improve the quality of embedding and sectioning of DRG neurons.

Histological Determination of Apoptosis

To determine the percentage of apoptotic cells, nuclear DNA fragments were tailed with digoxigenin-dUTP (Schmitz et al., 1991) and labeled with a fluorescein isothiocyanate (FITC) antibody as previously described (Gavrieli et al., 1993). Briefly, dissociated DRG on collagen-coated glass slides were fixed in 4% buffered formalin for 10 min at room temperature, washed in phosphate-buffered saline (PBS), and incubated with equilibration buffer, followed by freshly prepared terminal deoxynucleotidyl transferase (TdT) medium in a humid holding tray at 37°C for 1 h. After stopping the reaction with stop/wash buffer, cells were stained with freshly prepared anti-digoxigenin fluorescein (Oncor, Gaithersburg, MD) diluted as per the manufacturer's instructions, for 45 min at room temperature. The cells were then counterstained with 1 $\times$ freshly prepared propidium iodide for 15 min and mounted in Prolong (Molecular Probes, Eugene, OR) to preserve the immunofluorescent emission. Positive controls using DNA nicked with DNAse 1, and negative controls with water substituted for TdT were used for each experiment.

TEM

For TEM, five to six whole DRG were cultured per dish, with a total of 30–40 DRG/condition. DRG were fixed in fresh Trump's fixative (1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer) for a minimum of 2 h. Tissue was rinsed for 30 min in three changes of 0.15 M cacodylate buffer, pH 7.2, followed by 1 h postfix in 1% OsO_4. After rinsing in three changes of distilled water for 30 min, the tissue was en bloc stained with 2% uranyl acetate for 30 min at 60°C. After en bloc staining, the tissue was rinsed once in distilled water, dehydrated in progressive concentrations of ethanol, washed in 100% propylene oxide, and embedded in Spurr's resin. Thin (90-nm) sections were cut, placed on 200-μm mesh copper grids, and stained with lead citrate. All chemicals were obtained from Electron Microscopy Services (Ft. Washington, PA). Photographs were taken with the operator unaware of the treatment condition, using a Phillips CM100 TEM operating at 60 kV. Images were stored digitally.

Effect of NGF Withdrawal and IGF-I on Neuronal Apoptosis

To initiate NGF withdrawal, one of two methods was used. In both methods, the cultures were preplated on Primaria plates to remove most of the contaminating primary Schwann cells and fibroblasts (Deckwerth and Johnson, 1994). First, at the time of plating, dissociated DRG neurons were cultured in SFDM alone without NGF, or with NGF, IGF-I, or a combination of the two. Second, DRG neurons after preplating were cultured in SFDM, 10 ng/mL NGF, and 40 μM 5-fluoro-2' deoxyuridine (FUDR)/uridine (Sigma) to ensure removal of virtually all Schwann cells and fibroblasts. Cultures were maintained in FUDR/uridine for 5 days. The neuron rich cultures were then washed three times in EMEM and incubated in SFDM without NGF, NGF and IGF-I, or IGF-I alone. In cultures in which NGF was excluded, anti-NGF antibody (Collaborative Biomedical Products, Bedford, MA) in a concentration of 10 μg/mL was added to bind traces of NGF remaining after washing. Test medium was changed every 24 h. IGF-I used at a concentration of 1–100 nM was a gift from Cephalon (West Chester, PA). For composite data analysis, results were standardized against control conditions. To determine the percentage of apoptotic nuclei, neurons were cultured for 12–72 h in test media, photographed with a Leitz Orthoplan microscope, and counted by an observer blinded to the condition using a random-order number grid system as previously described (Russell et al., 1995). Approximately 5000 neurons were plated per slide, four slides were reviewed per condition, and experiments were repeated at least in triplicate. Only positively identified neuronal nuclei on corresponding phase contrast images were counted to exclude potential artifactual staining. DRG neurons have rounded, phase-bright cell bodies with clear neurite extensions. For electron microscopy, whole DRG were plated in test media for 48 h and then prepared for electron microscopy as described below. Apoptosis on TEM was not quantified; however, three thin sections separated vertically by at least 60 μm (to avoid photographing the same nucleus twice) were studied from each of the blocks.

To determine if the neuroprotective effect of IGF-I in neuronal survival was mediated by activation of PI 3-kinase or by mitogen-activated protein (MAP)-kinase signaling, we studied the effect of specific inhibitors on apoptosis. Dissociated DRG neurons were cultured as indicated above. Cultures were then incubated for 48 h in control medium, medium containing no NGF, with 100 nM IGF-I alone, with 100 nM IGF-I and 10 μM PI 3-kinase inhibitor (LY294002), or with 100 nM IGF-I and 10 μM MAP-kinase/extracellular signal-regulated protein kinase (ERK) inhibitor (PD98059). Both LY294002 and PD98059 were obtained from Biomol (Plymouth Meeting, PA). Cells were preincubated for 1 h with LY294002 or PD98059, prior to addition of IGF-I.

To determine if inhibition of caspase activation would inhibit apoptosis induced by NGF withdrawal, we used Boc-aspartyl (OMe)-fluoromethylketone (BAF), which generally inhibits caspases including caspase-3. DRG neurons were cultured as indicated above and then incubated for 48 h in control medium, medium containing no NGF, with 100 nM IGF-I alone, 100 μM BAF, or 100 μM BAF with 100 nM IGF-I. BAF was a gift from Dr. Brenda Shiver, Parke Davis, Ann Arbor, Michigan. Previous experiments in our laboratory indicated the optimal concentration of BAF for blocking PCD in DRG neurons was 100 μM.

Immunohistochemistry, *In Situ* Hybridization, and Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Dissociated E15 rat DRG neurons were cultured for 24 h in SFDM with 40 μM FUDR (Sigma). After fixing in 4% paraformaldehyde and permeabilizing the neurons with Triton X-100 for 15 min each, they were incubated in 10 μg/mL of chicken IGF-IR immunoglobulin Y (IgY) polyclonal Ab (Upstate Biotechnology, Lake Placid, NY) for 6 h, followed by 7.5 μg/mL of goat anti-chicken secondary antibody and 50 μg/mL avidin-fluorescein (Santa Cruz Biotechnology, Santa Cruz, CA), all at room temperature. The IGF-I receptor distribution was then visualized using a Noran Confocal Microscope, fitted with Oz with Intervision (Noran Instruments, Madison, WI).

Human DRG were obtained at autopsy between 8 and 12 h after death in patients in whom there was no evidence of neuropathy or of disorders predisposing to disease of the DRG. The bodies had been previously cooled within 1 h of death. For RT-PCR, total RNA was isolated from human adult autopsy DRG by a single-step method

(RNA STAT-60; Tel-Test ''B''); RNA was quantified by spectrophotometry at 260 nm and its integrity was tested by formaldehyde/agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μg total RNA in a 20-μL reaction containing 5 mM MgCl$_2$, 1× PCR buffer, 1 mM deoxyribonucleotide triphosphates, 2.5 μM random hexamers, 1 U/μL RNAse inhibitor, and 2.5 U/μL Moloney murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies, Gaithersburg, MD). PCR was performed in a 50-μL reaction containing 10 μL of each of the mixtures from the RT reaction, 2 mM MgCl$_2$, 1× PCR buffer, 0.6 mM dNTPs, 0.15 μM each of 5′ and 3′ primers, and 1.25 U/50 μL of Native *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT). Amplification was performed for 30 cycles, each cycle consisting of 1 min denaturation at 95°C, 2 min annealing at 54°C, and 3 min extension at 72°C; an additional extension time of 7 min at 72°C was added at the end of the 30 cycles. Primers were identified using the Right Primer BioDisk Software (Biodisk, San Francisco, CA). IGF-I receptor sense and anti-sense primers (5′-GCCTTTCA-CATTGTACGCATC-3′ and 5′-CTCCCACTCATCAG-GAACGTACAC-3′) were selected from human cDNA sequences (GenBank accession no. M23102) (Ullrich et al., 1986). The respective rat primers were antisense: 5′-GGCAGACGTCACAGAATCGATGGT-3′ and sense: 5′-CATCCGCAACGACTATCAGCA-3′ (GenBank accession no. M27293) (Werner et al., 1989). The PCR products were analyzed on a 2.0% agarose gel. The specificity of the obtained band was ascertained by cloning the PCR product (TA cloning kit; Invitrogen, San Diego, CA) and sequencing it with M13 forward and reverse universal primers, by the chain termination method (Sanger et al., 1977). Primers detecting β-actin mRNA (5′-CAATGCCAGGGTACATGGTGGTG-3′ and 5′-GGATCCGACTTCGAGCAAGAGATGGCCAC) were used to normalize for the amount of cDNA in each reaction.

After autopsy the human fifth lumbar DRG was removed and then embedded in paraffin as previously described (Dyck et al., 1986). For *in situ* hybridization, freshly cut sections were dewaxed in xylene, then ethanol, and then PBS. The tissue sections were immersed in 0.2 M HCl for 20 min, washed in 0.1% diethylpyrocarbonate water (DEPC H$_2$O), followed by PBS, and treated with 4 μg/mL proteinase K in a humid 50°C oven for 30 min. The reactions were stopped by two washes of PBS/SSC, followed by fresh 0.1 M triethanolamine and acetic anhydride. After further washing in SCC, the tissues were incubated in prehybridization buffer at 50°C, and then hybridization performed in a humid 50°C oven using digoxigenin-labeled IGF-I receptor probes. Equal total concentrations of sense and antisense RNA probes were used on each tissue section. The digoxigenin-labeled human or rat IGF-I receptor RNA probes were prepared as outlined in the manufacturer's recommendations (Boehringer Mannheim, Mannheim, Germany) and as previously described (Leventhal et al., 1995; Werner et al.,

1989). The tissues were washed in SSC and a series of buffers (Boehringer Mannheim) as previously described (Viaene and Baert, 1995). Immunological detection of the IGF-I receptor was performed by incubation of the tissues with an antidigoxigenin antibody at 37°C for 30 min, rewashed, and incubated in a mixture of 4-Nitro blue tetrazolium chloride in buffer, 5-bromo-4-chloro-3-indolylphosphate, and 125 nM levamisole. After 3 h, the color reaction was stopped in Tris-HCl/ethylenediaminetetraacetic acid (EDTA) buffer. Control sections were hybridized either without labeled probe, with digoxigenin-labeled antisense Neo RNA (Boehringer Mannheim), or with labeled sense probe.

Statistical Analysis

Assumptions about the Gaussian distribution of data were made using normalized cumulative frequency polygons and determination of the skewness and kurtosis of data. Data not exactly corresponding to a Gaussian distribution were analyzed using an appropriate mathematical transformation, usually log (x). Comparison of dependent variables was performed using factorial analysis of variance (ANOVA), and Fisher's LSD test was used as a posterior procedure for making pairwise comparisons of means. Inference was made using a probability <.05. For graphic and descriptive purposes, data are expressed in actual units ± the standard error of the mean (S.E.M.).

RESULTS

IGF-I Protects Neurons from Apoptosis after NGF Withdrawal

When DRG neurons were cultured for 5 days in SFDM/FUDR and then NGF were withdrawn and 10 μg/mL NGF antibody was added (Fig. 1), there was an increase in the percentage of apoptotic neurons, ranging from 36.28 ± 5.30% at 12 h to 99.70 ± 0.21% at 72 h. By 48 h, most of the DRG neurons were dead (96.80 ± 1.40%). The percentage of apoptotic neurons cultured in control SFDM containing 10 ng/mL NGF increased from 1.29 ± 0.61% at 12 h to 10.72 ± 3.15% at 72 h. In contrast, the percentage of apoptotic neurons after NGF withdrawal, but in the presence of IGF-I (Fig. 1), was essentially unchanged or slightly reduced after 72 h. At 72 h, the percentage of apoptotic neurons was 5.10 ± 0.95 for 1 nM IGF-I and 1.93 ± 0.73 for 100 nM IGF-I. At all time periods including 72 h, the percentage of apoptotic neurons in the presence of IGF-I alone was dose dependent. At 12 h, the percentage of apoptotic neurons in control medium containing NGF was 8% less than with 1 nM IGF-I alone (Fig. 1), and was significantly
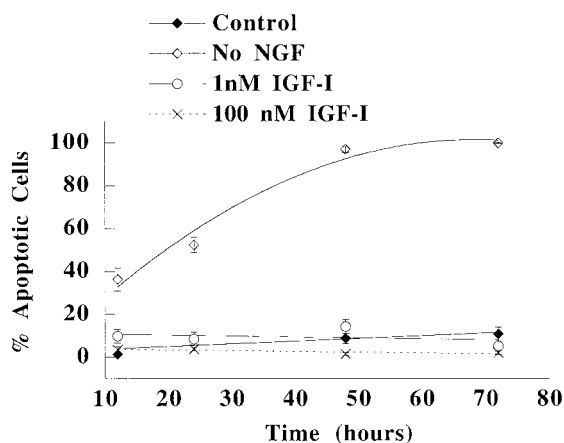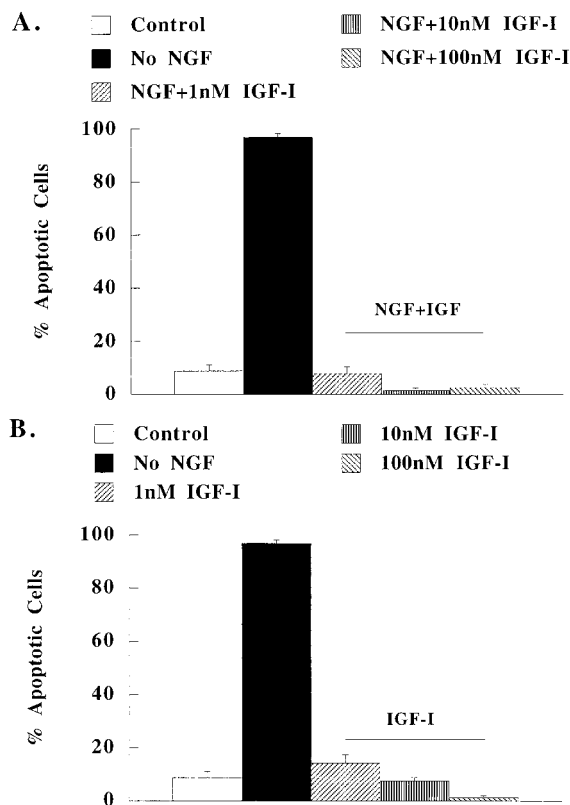
Figure 1 IGF-I prevents apoptosis in rat DRG neurons after NGF withdrawal. DRG neurons were cultured up to 72 h in SFDM containing 10 ng/mL NGF (control), no NGF with 10 μg/mL anti-NGF Ab, and 1–100 nM IGF with 10 μg/mL anti-NGF Ab. After NGF withdrawal, the percentage of apoptotic neurons increased up to 72 h, with most of the neurons dying by 48 h. In the presence of 100 nM IGF-I, <5% apoptotic neurons were seen at 72 h, less than in control neurons with NGF (p < .05). Neuronal survival in the presence of IGF-I alone, even at low concentrations (1 nM), at 12–72 h was statistically different from neurons in which NGF was withdrawn (p < .001).

different from 1 nM IGF-I (p < .05). However, at 12 h, there was no difference in the percentage of apoptotic cells in control medium containing NGF compared to medium containing 10 or 100 nM IGF-I, although there was a significant difference between 1 and 10 or 100 nM IGF-I (p < .05). In contrast, by 24 h, there was no statistical difference between the percent apoptotic neurons with control medium containing NGF and medium containing 1 nM IGF-I (Fig. 1).

When combined NGF/IGF-I and IGF-I alone were compared, we found that at 12 h there was 0.81 ± 0.29% apoptotic neurons with 10 ng/mL NGF and 1 nM IGF-I compared to 9.83 ± 3.08% with 1 nM IGF-I alone. However by 24 h, combined NGF and 1 nM IGF-I, or NGF and ≥10 nM IGF-I at any time point (data not shown) did not further significantly decrease the percentage of apoptotic neurons, suggesting that the antiapoptotic effects of NGF and IGF-I are not additive.

Dorsal root ganglion neurons were cultured in SFDM/FUDR as above, NGF were withdrawn, 10 μg/mL NGF antibody was added, and then test medium was added for 48 h (Fig. 2). Under these conditions, we found that most NGF deprived

DRG neurons had undergone apoptosis [Fig. 2(A)]. IGF-I inhibited apoptosis after NGF withdrawal in a dose-dependent fashion in that the percentage of apoptotic neurons decreased from 14% with 1 nM IGF-I to 7.6% with 10 nM IGF-I, and 1.4% with 100 nM IGF-I [Fig. 2(B)]. The percentage of apoptotic neurons was significantly different with 1 nM IGF-I alone compared to 10 nM IGF-I alone (p < .01) and 100 nM IGF-I (p


Figure 2 IGF-I prevents apoptosis of rat DRG neurons after NGF withdrawal at 48 h. (A) DRG neurons were cultured for 48 h in SFDM with 10 ng/mL NGF, without NGF, and with NGF and 1–100 nM IGF-I combined. (B) DRG neurons cultured as above with 1–100 nM IGF-I alone. Condition 1 and 2 are the same in (A) and (B). In the absence of NGF, >95% of neurons were dead at 48 h. The percentage of apoptotic neurons was reduced by the addition of 1 nM IGF-I to 14% (p < .0001), and the number of apoptotic neurons was further reduced in a dose-dependent manner to 7.6% with 10 nM IGF-I, and 1.4% with 100 nM IGF-I. Although there was a slight decrease in the percentage of apoptotic neurons when NGF was combined with IGF-I, there was no statistical significance between neurons treated with IGF-I alone compared to NGF and IGF-I. The values are representative of the mean of three separate experiments, standardized against control values.

< .01), consistent with a dose-dependent increase in IGF-I neuroprotection against apoptosis after NGF withdrawal. In the presence of 100 nM IGF-I alone [Fig. 2(B)], the percentage of apoptotic neurons was significantly less than in control medium with NGF ($p < .05$).

In a separate set of experiments, NGF was withdrawn or IGF-I added from culture day 1 instead of culture day 6. No anti-NGF antibody was used in these experiments, but cultures were pre-plated to remove contaminating Schwann cells and fibroblasts. In these shorter-term experiments, at 48 h the percentage of apoptosis in control neurons (15.85 ± 5.33%) was slightly higher than longer-term cultures, but 100 nM IGF-I alone with no NGF still reduced the percentage of apoptosis to 6.77 ± 2.90%. As with the longer-term cultures described above, IGF-I was neuroprotective in a dose-dependent manner after NGF withdrawal, and there was no additive effect with NGF and IGF-I combined (data not shown). Thus, although apoptosis was greater in controls in the first 2 days in culture compared to that after 1 week in culture, the neuroprotective effect of IGF-I was similar.

The classical changes of apoptosis were observed in DRG neurons after NGF withdrawal. Neurons were cultured in SFDM/FUDR for 48 h after preplating, and then test medium was added for 48 h. Anti-NGF antibody (10 μg/mL) was added in addition to NGF withdrawal. Condensation of the nuclear chromatin into apoptotic bodies, and shrinkage of the nucleus and cytoplasm, but preservation of the cytoplasmic membrane were observed. At 48 h, almost all neurons showed evidence of apoptosis, and there was single deletion of DRG neurons with preservation of surrounding satellite cells and Schwann cells [Fig. 3(B)]. With IGF-I alone [Fig. 3(C,D)], there was sparing of neurons. Although with 1 nM IGF-I the neuronal nucleus [Fig. 3(C)] showed normal chromatin distribution, there was evidence of minimal perikaryeal shrinkage. With low-dose IGF-I, some of the neurons continued to classical changes of apoptosis, whereas with high-dose IGF-I (100 nM), most of the DRG neurons were morphologically normal with TEM. With combined NGF and IGF-I [Fig. 3(E,F)], both DRG neurons and satellite cells were morphologically normal. With NGF and 1 nM IGF-I, neuronal perikarya were less likely to show early apoptotic changes compared to 1 nM IGF-I alone, but no difference was seen with 100 nM IGF-I compared to NGF and 100 nM IGF-I.
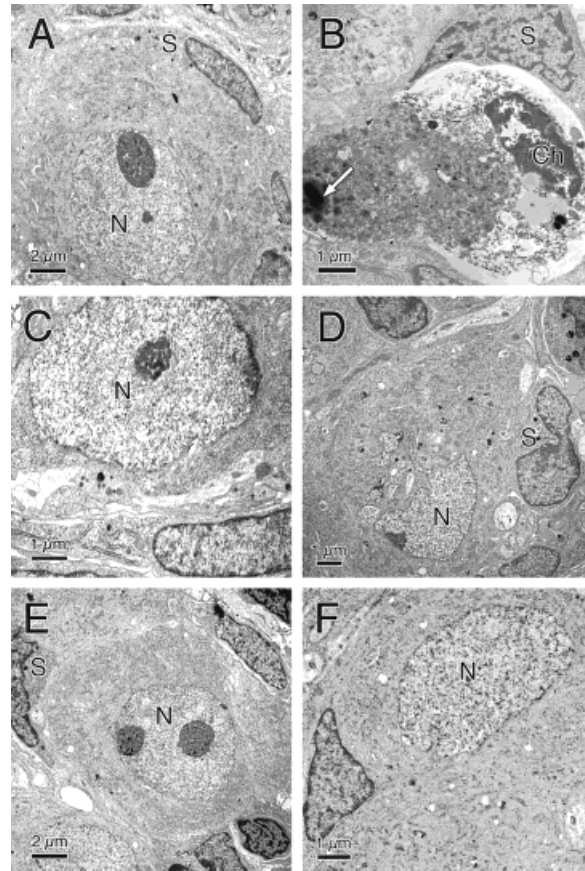
Figure 3 TEM of *in vitro* rat DRG at 48 h showing neuronal rescue by IGF-I in the absence of NGF. (A) Control DRG with 10 ng/mL NGF showing normal chromatin staining in the nucleus (N), and an intact perikaryon. The neuroblast was encased in satellite cells (S). (B) DRG after 48 h NGF withdrawal. There was severe chromatin clumping (Ch) observed in the nucleus, shrinkage of the perikaryon—although at higher magnification the plasma membrane remained intact—and single deletion of cells. At 48 h, most of the neuroblasts were apoptotic. There was also a prominent collection of lipofusion (white arrow) commonly observed in apoptotic DRG neurons. The satellite cells (S) were histologically unaffected by the NGF withdrawal. (C) DRG with 1 nM IGF-I, but no NGF. The presence of IGF-I supported neuronal survival as indicated, although several neuroblasts continued to undergo apoptosis at this low concentration of IGF-I. (D) DRG with 100 nM IGF-I. There was survival of most of the neuroblasts, with normal chromatin distribution in the nucleus (N). The normal neuronal perikaryon was surrounded by satellite cells (S). (E) DRG with 1 nM IGF-I and 10 ng/mL NGF. (F) DRG with 100 nM IGF-I and 10 ng/mL NGF. In both (E) and (F), the neuronal nuclei (N) were normal. Apoptotic neurons were rare in the presence of both NGF and IGF-I, and survival was slightly greater than controls at higher concentrations of IGF-I.

Table 1 IGF-I and Neuronal Cell Death

Condition	% Apoptosis	S.E.M.	Condition	% Apoptosis	S.E.M.
NGF 10 ng/mL	8.82	3.50	LY294002 + IGF-I	99.20	0.25
No NGF	95.40	2.40	PD98059 + IGF-I	3.40	0.58
IGF-I 100 nM	0.22	0.11	BAF	0.34	0.19
			BAF + IGF-I	0.38	0.35

NGF: nerve growth factor; IGF-I: insulin-like growth factor I; LY294002: PI 3-kinase inhibitor; PD98059: MAP kinase inhibitor; BAF: Boc-aspartyl (OMe)-fluoromethylketone, a caspase inhibitor; S.E.M.: standard error of the mean.

IGF-I Prevents Neuronal Apoptosis after NGF Withdrawal by Activation of PI 3-Kinase Signaling

The neuroprotective effect of IGF-I after NGF withdrawal was blocked by 10 μM LY294002, an inhibitor of PI 3-kinase (Table 1). Since LY294002 will also potentially block NGF-induced PI 3-kinase signaling, we examined whether LY294002 would block PI-3 kinase signaling induced specifically by IGF-I alone. In the presence of 100 nM IGF-I alone, most neurons survived NGF withdrawal at 48 h. However with 10 μM LY294002 and 100 nM IGF-I, 99% of neurons were dead at 48 h ($p < .0001$). In the presence of 10 μM PD98059, an inhibitor of MAP-kinase/MEK signaling, 100 nM IGF-I still reduced the percentage of apoptotic neurons to below control levels ($p < .05$), although IGF-I and PD98059 were slightly less neuroprotective than IGF-I alone (not statistically significant). Similar results were obtained whether or not anti-NGF antibody was present in test medium. We had previously shown in neurons that concentrations of PD98059 >10 μm had no appreciable additive effect in blocking the dual-specificity protein kinase MEK (Kim et al., 1997), and in preliminary data did not further block MAP kinase signaling in DRG neurons.

Inhibition of the Caspase PCD Pathways Inhibits Apoptosis Induced by NGF Withdrawal

Inhibition of caspase cleavage by BAF (100 μM) almost completely blocked apoptosis induced by NGF withdrawal at 24 and 48 h. When DRG neurons were cultured for 5 days in NGF, BAF was added, NGF was withdrawn and 10 μg/mL anti-NGF antibody was added, the percentage of apoptotic neurons was 0.34 ± 0.19% compared to 8.82 ± 3.50% control neurons ($p < .05$). The percentage of apoptotic neurons could not be further decreased by the addition of 100 nM IGF-I to 100 μM BAF, and results were not statistically significantly differ-

ent from IGF-I alone or BAF alone. Apoptosis was less completely blocked by 10 μM BAF (data not shown).

IGF-I Receptor Distribution in Rat and Human DRG

Dorsal root ganglion neurons were found to express functional IGF-I receptor during development (E15 rat) and also in the adult human using both RT-PCR and *in situ* hybridization techniques (Figs. 4 and 5). The sizes of the PCR products for human and rat IGF-I receptor were, respectively, 600 and 933 bp. Specificity was confirmed by sequencing the PCR products and finding 98% homology with predicted sequences (Werner et al., 1989; Ullrich et al., 1986). Using immunohistochemistry with antibody specific for the IGF-I receptor, we demonstrated that the IGF-I receptor was abundantly distributed throughout the DRG neuron [Fig. 5(A)]. With confocal microscopy and z-series resolution of the three-dimensional structure of the neuron, we
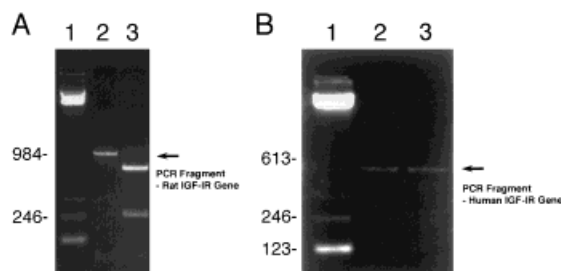
Figure 4 RT-PCR analysis of RNA transcripts using primers from IGF-I receptor in E15 embryonic rat DRG neurons (A) and human DRG neurons (B). (A) Rat DRG: Lane 1 contains a 123-base pair (bp) DNA ladder, and lane 2, an RT-PCR product from IGF-I receptor mRNA; lane 3, a *Ban1* digest of lane 2 product yielding predicted 246- and 684-bp products. (B) Human DRG: Lane 1 contains a 123-bp DNA ladder; a 600-bp RT-PCR fragment from adult human mRNA is seen in lanes 2 and 3 (two separate samples). Equal levels of β-actin were identified in each of the RT-PCR samples.
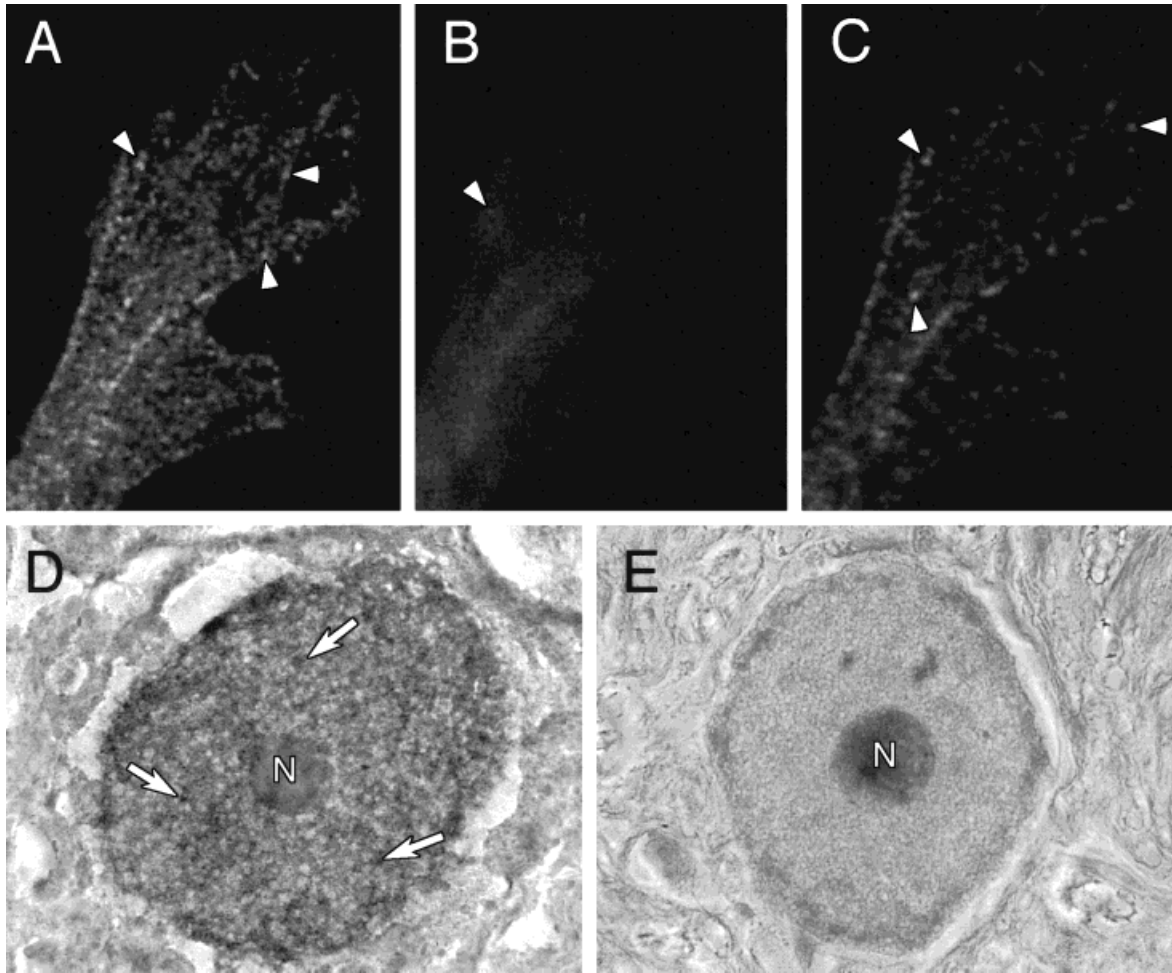
Figure 5   IGF-I receptor expression in the rat DRG neurite growth cone and in human DRG. (A)   The IGF-I receptor (white arrows) was abundant in the rat neurite growth cone. Rat DRG were incubated with chicken IGF-IR IgY and viewed using confocal microscopy ($\times$1500). The figure represents a z-series reconstruction of several image planes from the upper to the lower surface of the neurite growth cone. The IGF-I receptor was distributed throughout the neuron, but was most abundant in the DRG growth cone. (B)   Image from an upper plane from the z-series described in (A). The image plane was adjusted to lie just below the upper surface of the neuron. Although the outline of the growth cone is seen, there are few IGF-I receptors present. (C)   Image from a lower plane (adjacent to the ECM) of the z-series described in (A). More numerous IGF-I receptor staining (white arrows) is observed throughout the growth cone. (D)   *In situ* hybridization for IGF-I receptor in human DRG using antisense probe showing abundant punctate labeling of IGF-I receptor mRNA (white arrows) throughout the neuronal perikaryon ($\times$500). N = DRG neuronal nucleus. There was less abundant labeling of satellite cells and fibroblasts. (E)   Sense control showing no labeling of the neuron or supporting cells ($\times$500). N = DRG neuronal nucleus. Equal total concentrations of sense and antisense RNA probes were used on each tissue section.

found that the IGF-I receptor was mainly distributed near the surface of the neuron, was more abundant in the region of the neurite growth cone, and extended out to the outer edges of the developing lamellipodia [Fig. 5(A)]. Using z-series reconstruction, the receptor distribution was greater on the surface of the neuron in contact with the extracellular matrix (ECM), although the IGF-I receptor could be demonstrated throughout the neuron.

In human DRG, *in situ* hybridization using specific RNA probes to human IGF-I receptor mRNA developed with antidigoxigenin antibody showed

abundant signal throughout the neuronal cytoplasm and was not localized to a specific region of the perikaryon [Fig. 5(B)]. The signal was likely specific as indicated by the absence of signal to labeled sense RNA probes [Fig. 5(C)]. In rat DRG, IGF-I receptor mRNA was similarly widely distributed throughout the neuronal perikaryon (data not shown). In adult human DRG, IGF-I receptor mRNA was less abundant in satellite cells and fibroblasts.

DISCUSSION

In this study, we examined the ability of IGF-I to prevent apoptosis in DRG neurons after NGF withdrawal. Apoptosis refers to the characteristic morphological changes observed in PCD, including deletion of single cells, cell shrinkage, membrane blebbing, and compaction of chromatin (Kerr et al., 1972; Majno and Joris, 1995; Raff, 1992). Similar to sympathetic neurons, when NGF was withdrawn from DRG, the majority of neurons were committed to die by 24 h (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994) and apoptosis was the predominant form of cell death (Edwards and Tolkovsky, 1994). Using TEM, we observed prominent chromatin condensation of DRG neuronal nuclei, shrinkage of the perikaryon, and retention of plasma membrane. In addition, many of the apoptotic DRG had more prominent accumulations of lipofuscin granules in the cytoplasm than control neurons, a change that has been associated with aging and degeneration of neurons (Kerezoudi et al., 1995; Sasaki et al., 1997).

Insulin-like growth factor-I prevented apoptosis in DRG sensory neurons for at least 72 h after NGF withdrawal. In the presence of high concentrations of IGF-I, most of the DRG neurons had a normal histological appearance on TEM, and even small concentrations of IGF-I (1 nM) maintained viability of neurons in the absence of NGF. We also used TdT-mediated dUTP-biotin nick end labeling (TUNEL) to quantitate the number of apoptotic neurons. In these experiments, we took advantage of the fact that in our culture system we can study a nearly pure neuronal population. Under these conditions, the calculated percentage of apoptotic neurons corresponded with the histological findings on TEM. IGF-I alone maintained neuron viability in the absence of NGF for periods of at least 72 h. Furthermore, although neuron survival was improved slightly by combining low-dose IGF-I with NGF, at higher concentrations of IGF-I concomitant NGF

had no additional antiapoptotic effect, suggesting that NGF and IGF-I do not necessarily act additively in inhibiting PCD. When IGF-I is used *in vitro*, as with neurotrophic factors such as NGF (Russell et al., 1994), the concentration of IGF-I required to promote a maximum biological effect is often greatly in excess of the receptor dissociation constant for that factor. Although an almost maximal antiapoptotic response was seen with 10 nM IGF-I, a small additive effect was achieved with 100 nM IGF-I. These observations may relate to IGF-binding proteins (IGFBPs) modulation of IGF-I binding at the IGF-I receptor (see Jones and Clemmons, 1995, for review), uptake and internalization of IGF-I by the IGF-I receptor, or proteolysis of IGF-I within the culture system.

Nerve growth factor withdrawal has been well described as a model for PCD in sympathetic neurons (see Deshmukh and Johnson, 1997, for review), as well as early differentiating cerebellar granule neurons (Muller et al., 1997), developing basal forebrain cholinergic neurons (Kew and Sofroniew, 1997), and differentiated PC12 cells (Tong et al., 1997). Recently, it has been shown that inhibition of trk A receptor tyrosine signaling does not affect apoptosis occurring in axotomized mouse DRG and nerve stumps. Furthermore, addition of NGF did not reduce the percentage of apoptotic neurons (Edstrom et al., 1997). This suggests that NGF may have a more circumscribed role in preventing PCD in DRG than initially proposed (Lindsay, 1988), whereas other growth factors like IGF-I may be important. In parallel with the current studies, we (Matthews and Feldman, 1996; Russell et al., 1996; Singleton et al., 1996a) and others (Miller et al., 1997) also found that IGF-I prevents PCD owing to a variety of neurotoxic stimuli in both transformed and primary neural cell lines.

Signaling through the PI 3-kinase pathway is important in the prevention of apoptosis by NGF (Yao and Cooper, 1995). We found that inhibition of PI 3-kinase signaling by LY294002 blocks the antiapoptotic effect of IGF-I after NGF withdrawal in sensory neurons. In comparison, the MAP-kinase/ERK inhibitor PD98059 had little effect on neuronal apoptosis within this paradigm. We and others previously reported that PI 3-kinase signaling in neuronal and nonneuronal cells is important for IGF-mediated inhibition of PCD from a variety of proapoptotic stimuli including high glucose and hyperosmolarity (Singleton et al., 1996a; Russell et al., 1997; Párrizas et al., 1997; D'Mello et al., 1997; Kulik et al., 1997); in contrast, neurite outgrowth *in vitro* requires signaling though the MAP-kinase

pathway (Kim et al., 1997). While our current and past data suggest IGF-I mediates separable signaling pathways effecting cell death, Párrizas et al. (1997) reported an additional role for MAP-kinase/ERK signaling in IGF-I inhibition of apoptosis occurring in PC12 cells after NGF deprivation.

We have previously found that under conditions of hyperglycemic or hyperosmolar stress autophosphorylation of IGF-IR activates PI 3-kinase, which in turn up-regulates protein expression of the PCD inhibitors bcl-2 and bcl-xL (Singleton et al., 1996a; Russell et al., 1997). These proteins determine whether a ''death switch'' is activated. Once activated, this switch turns on caspase-8 and other downstream ced-3/interleukin-1β converting enzyme (ICE)-like proteases, such as caspase-3 (pro-Yama and pro-CPP32) (Chinnaiyan et al., 1996; Singleton et al., 1996a; Russell et al., 1997). In sympathetic neurons, NGF and inhibitors of the ICE-like proteases block apoptosis at a similar time point (Deshmukh et al., 1996). The small, cell-permeable, aspartate-containing ''pan-caspase'' inhibitor BAF (Deshmukh and Johnson, 1997) has been shown to inhibit cleavage of caspase-3 *in vitro.* This inhibitor contains sequences corresponding to the aspartate-containing cleavage site of all caspases. We found that BAF was able to almost completely block PCD in DRG neurons after NGF withdrawal, an effect that was maintained for at least 48 h. The degree of PCD inhibition by BAF was similar to that observed with IGF-I alone, and combining IGF-I and BAF did not produce additive inhibition of apoptosis. In agreement with our results, two other peptide inhibitors of ICE, Ac-YVAD-CHO and Ac-YVAD-CMK, are able to block PCD in motor neurons deprived of trophic factors (Deshmukh and Johnson, 1997). Collectively, these results, along with our previous observations on IGF-I–mediated inhibition of PCD (Russell et al., 1996, 1997; Singleton et al., 1996a), indicate that IGF-I, like BAF, is able to inhibit activation of the caspase death cascade after NGF withdrawal. However, unlike BAF, IGF-I interrupts cell death signaling well upstream of caspase activation (Russell et al., 1996, 1997; Singleton et al., 1996a).

The distribution of the IGF-I receptor has been previously described both *in vivo* and *in vitro* within the mammalian brain (Han et al., 1987) and in avian brain and sensory ganglia (Holzenberger et al., 1997). We found that both human and rat DRG abundantly express IGF-I receptor mRNA throughout the DRG neuronal perikaryon. *In vitro,* the IGF-I receptor was present in rat sensory neurons, and using confocal imaging appeared to be concentrated on the extracellular matrix surface of the growth cone. Our results agree with previous work suggesting the IGF-I receptor is more abundant in neurons than in glia (Burgess et al., 1987) and at growth cones within the brain (Mascotti et al., 1997). The abundance of IGF-I receptor within the growth cone suggests a role for IGF-I in axonal adhesion and structural rearrangement of the cytoskeleton. We recently found that IGF-I promotes remodeling of the actin cytoskeleton and formation of focal adhesions via activation of PI-3 kinase (Leventhal et al., 1997; Leventhal and Feldman, 1997). These data are interesting in light of evidence suggesting an association between PCD, cellular adhesion, and the cytoskeleton (Malorni et al., 1995). Indeed, cells denied anchorage undergo a form of PCD called ''anoikis'' (Frisch and Francis, 1994). Attachment per se is insufficient to prevent anoikis; what is required is the tyrosine phosphorylation of kinases such as focal adhesion kinase (FAK) involved in cytoskeletal organization (Ruoslahti and Reed, 1994; Topper et al., 1995). Collectively, these findings suggest that the DRG IGF-I receptor may, in addition to its effect on bcl/caspase signaling, prevent PCD by promoting adhesion to the extracellular matrix and stabilization of the actin cytoskeleton.

In summary, the abundance of functional IGF-I receptor both in embryonic DRG and adult human DRG suggests an important role for IGF-I in sensory neurons. IGF-I's primary effect on embryonic DRG sensory neurons is inhibition of PCD after NGF withdrawal. Inhibition of PCD requires IGF-I directed signaling through the PI 3-kinase pathway, but not through MAP kinase/ERK. Our results support a role for IGF-I in DRG embryogenesis, and suggest that IGF-I may afford a potential treatment for neurotoxic and traumatic neuropathies.

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