Programmed cell death in the developing inner ear is balanced by nerve growth factor and insulin-like growth factor I

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Accepted 14 October 2002
Journal of Cell Science 116, 475-486 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00223

Summary

Nerve growth factor induces cell death in organotypic cultures of otic vesicle explants. This cell death has a restricted pattern that reproduces the in vivo pattern of apoptosis occurring during inner ear development. In this study, we show that binding of nerve growth factor to its low affinity p75 neurotrophin receptor is essential to achieve the apoptotic response. Blockage of binding to p75 receptor neutralized nerve-growth-factor-induced cell death, as measured by immunoassays detecting the presence of cytosolic oligonucleosomes and by TUNEL assay to visualize DNA fragmentation. Nerve growth factor also induced a number of cell-death-related intracellular events including ceramide generation, caspase activation and poly-(ADP ribose) polymerase cleavage. Again, p75 receptor blockade completely abolished all of these effects. Concerning the intracellular pathway, ceramide increase depended on initiator caspases, whereas its actions depended on both initiator and effector caspases, as shown by using site-specific caspase inhibitors. Conversely, insulin-like growth factor I, which promotes cell growth and survival in the inner ear, abolished apoptosis induced by nerve growth factor. Insulin-like growth factor cytoprotective actions were accomplished, at least in part, by decreasing endogenous ceramide levels and activating Akt. Taken together, these results strongly suggest that regulation of nerve-growth-factor-induced apoptosis in the otocysts occurs via p75 receptor binding and is strictly controlled by the interaction with survival signalling pathways.

Key words: p75 neurotrophin receptor ($p75^{NTR}$), Ceramide, Caspase activation, Cell survival, Otic vesicle, Ceramide-1-phosphate

Introduction

Programmed cell death is a critical process for normal development and tissue homeostasis (Glücksmann, 1951; Raff, 1992; Jacobson et al., 1997; Vaux and Korsmeyer, 1999). Although the basic program of apoptosis execution remains conserved, distinct regulatory signals have been described depending on the cell type and developmental stage (D'Mello, 1998). Particularly interesting are the opposite actions displayed by nerve growth factor (NGF) that can act either as a survival factor or as a death-inducing factor (Casaccia-Bonnefil et al., 1999; Lee et al., 2001). A coherent understanding of the regulation of programmed cell death during development requires a coordinated study of the multiple signals acting on specific cells.

Inner ear ontogenesis is an attractive model system to analyse which signals are implicated in the modulation of cell death and survival. The vertebrate inner ear originates from the head ectoderm where the otic placode is formed and invaginates to form the otic pit that later closes forming the otic vesicle or otocyst. This is a transient embryonic structure that undergoes a distinct period of intense cell proliferation [stages 18-22 in the chicken (Hamburger and Hamilton, 1951)]. This proliferation precedes the differentiation of the various cell types and compartments that will later form the adult inner ear (Bissonnette and Fekete, 1996). In parallel, neuroblasts for the cochleo-vestibular ganglion (CVG) migrate out from the medial wall of the otic vesicle. The CVG contains the afferent neurons that connect the sensory epithelium of the inner ear to the central nervous system (Hemond and Morest, 1991). Concomitantly with these biological processes, in the otic primordium and CVG there are areas of intense programmed cell death (Alvarez and Navascués, 1990; Sanz et al., 1999a). Complementary in vivo and in vitro studies point to insulin-like growth factor-I (IGF-I) and NGF as two key diffusible factors operating during inner ear development (reviewed by Torres and Giraldez, 1998; Frago et al., 2000).

NGF and the related neurotrophins are essential for cell survival and maintenance of the nervous system during development (Bibel and Barde, 2000; de la Rosa and de Pablo, 2000). The role of neurotrophins in neuronal survival is mainly mediated by the activation of high-affinity Trk receptors (Yoon et al., 1998; Klesse and Parada, 1999). However neurotrophins

also bind the low-affinity p75 neurotrophin receptor ($p75^{NTR}$), which is a modulator of survival and death decisions (Casaccia-Bonnefil et al., 1999; Barrett, 2000; Lee et al., 2001). The $p75^{NTR}$ is structurally related to well known death receptors, that is, the tumour necrosis factor-α (p55) and Fas superfamily of receptors. Similarly, $p75^{NTR}$ possesses a death domain that upon binding to ligand elicits the intracellular responses that culminate in cell death (Beutler and van Huffel, 1994; Liepinsh et al., 1997). $p75^{NTR}$-knockout mice present severe nervous system defects (von Schack et al., 2001). In the past few years, the role of NGF as an inducer of apoptosis has become evident. For example, treatment with NGF causes cell death in cultures of mature oligodendrocytes (Casaccia-Bonnefil et al., 1996) and in the developing chicken retina via an undefined mechanism mediated by $p75^{NTR}$ (reviewed in Frade and Barde, 1998). NGF also induces apoptotic cell death in organotypic cultures of otic vesicles (Frago et al., 1998). Interestingly, NGF-induced cell death occurs in specific areas of the otic vesicle and CVG, suggesting that during early inner ear organogenesis there is strict control of NGF actions. The presence of $p75^{NTR}$ has been reported at different stages of inner ear development in several animal species, including chicken (Hallbook et al., 1990; von Bartheld et al., 1991; Schecterson and Bothwell, 1994; Wu and Oh, 1996). The precise signalling pathway(s) used by $p75^{NTR}$ to activate cell death remain unclear, but they may involve generation of ceramide (Dobrowsky et al., 1994; Frago et al., 1998; Casaccia-Bonnefil et al., 1996; Lievremont et al., 1999) and activation of caspases-1, -2 and -3 (Gu et al., 1999) as well as cyclin-dependent kinases (Frade, 2000).

IGF-I is a pleiotropic factor for the epithelial and neuronal cells of the inner ear, which is where IGF-I and its type 1 receptor are expressed throughout development (León et al., 1999). The importance of IGF-I in ear development is stressed by the fact that deficiency in IGF-I results in sensorineural deafness in humans (Woods et al., 1996; Woods et al., 1997). Likewise, the lack of IGF-I in mice severely affects postnatal survival, differentiation and maturation of the cochlear ganglion cells and causes abnormal innervation of the sensory cells in the organ of Corti (Camarero et al., 2001). Furthermore, IGF-I is a survival and growth factor capable of blocking apoptosis in organotypic cultures of chick otic vesicles (Frago et al., 1998). By binding to its high-affinity tyrosine kinase receptor, IGF-I activates the Raf/MAPK cascade and induces the expression of Fos, Jun and proliferating cell nuclear antigen (PCNA), which leads to cell growth (Frago et al., 2000; Sanz et al., 1999b). However, little is known about the intracellular pathways elicited by IGF-I to promote cell survival during development. One of the pathways by which IGF-I exerts its anti-apoptotic effect in different cell types including cells of the nervous system is the activation of the Akt/Protein kinase B pathway (Dudek et al., 1997). Akt family members have been shown to be required for normal development in flies and *Caenorhabditis elegans* (Paradis and Ruvkun, 1998; Verdu et al., 1999), and Akt1-null mice present defects in both foetal and postnatal growth (Cho et al., 2001).

Here we have explored the intracellular signalling mechanisms elicited by NGF to induce apoptosis in the developing inner ear and how they are balanced by survival signals activated by IGF-I. We show that (i) $p75^{NTR}$ mediates NGF pro-apoptotic effects, including ceramide generation, caspase activation and DNA fragmentation; (ii) NGF induces ceramide generation via synthesis de novo and acid sphingomyelinase activation; (iii) NGF induces the activation of initiator and effector caspases and the hydrolysis of poly-(ADP ribose) polymerase (PARP); (iv) only initiator caspases are involved in ceramide generation; and (v) IGF-I prevents NGF actions, at least in part, by decreasing intracellular ceramide levels and activating Akt.

Materials and Methods

Materials

Human recombinant IGF-I was purchased from Roche Molecular Biochemicals (Basel, Switzerland). Human recombinant IGF-I is equipotent with chicken IGF-I when assayed in organotypic cultures of chicken otic vesicles (León et al., 1998). Mouse 2.5S NGF was routinely evaluated on chick embryo dorsal root ganglia (UBI, Lake Placid, NY). Phosphatidic acid, from Sigma (St Louis, MO), was handled as reported previously (Kishikawa et al., 1999). The antibody to PCNA was from Concepta Biosystems, SA (Barcelona, Spain). Anti-NGF mouse monoclonal antibody was from Roche Molecular Biochemicals. The anti-$p75^{NTR}$ antibody utilised for immunocytochemistry was from Santa Cruz Biotechnology (Santa Cruz, CA). The blocking anti-$p75^{NTR}$ rabbit polyclonal antiserum (9651) was made against the extracellular domain of mouse $p75^{NTR}$, whereas the non-blocking anti-$p75^{NTR}$ antibody (9992) recognises the intracellular domain. The anti-$p75^{NTR}$ 9651 antibody blocks binding of 125I-NGF to rat or mouse $p75^{NTR}$ in affinity crosslinking assays (1:100 dilution) (Huber and Chao, 1995). Both antibodies were kindly provided by M. V. Chao (Skirball Institute of Biomolecular Medicine, New York University School of Medicine). The anti-PARP rabbit polyclonal antibody was from Chemicon International Inc. (Temecula, CA) and recognises both 116 kDa PARP and the apoptosis-related cleavage fragment of 85 kDa. Anti-phospho-Akt (Ser473 and Thr308) rabbit polyclonal antibodies were from New England Biolabs (Beverly, MA) and anti-Akt goat polyclonal antibody was from Santa Cruz Biotechnology Inc. Secondary antibodies conjugated with horseradish peroxidase were purchased from Bio-Rad (Hercules, CA). C_2-ceramide (C_2-Cer) was from Matreya Inc. (Pleasant Gap, PA), whereas inactive C_2-dihydroceramide and C_8-ceramide-1-P (Cer-1-P) were from Calbiochem (San Diego, CA). We used the t.l.c. system described below to routinely test the presence of ceramide in commercial Cer-1-P batches. The activity of the batches was tested in parallel for the presence of oligonucleosomes in the cytosol, which was taken as an index of DNA fragmentation. Only batches of ceramide-free Cer-1-P were used. Cer-1-P was prepared in water and sonicated before its addition to culture medium. [^{3}H]palmitic acid (5 mCi/ml) was from Amersham Ltd. (Little Chalfont, England). Fumonisin B1 (FB1), an inhibitor of ceramide synthesis de novo (Herget et al., 2000), and desipramine, an inhibitor of acid sphingomyelinase (Chatterjee and Wu, 2001), were from Sigma. Manumycin A, an irreversible inhibitor of neutral sphingomyelinase (Arenz et al., 2001), was from Calbiochem. R59949, an inhibitor of diacylglycerol kinase (DGK), was from Calbiochem (San Diego, CA) (Jiang et al., 2000).

The caspase inhibitors, zVAD-fmk (zVAD) (Calbiochem) and z-DEVD-fmk (DEVD) (Enzyme Systems Products, Livermore, CA), contain a peptide recognition sequence attached to a functional fluoro-methylketone (fmk) group. These inhibitors are cell permeable and act by binding irreversibly to the active site of caspases. zVAD is a pan-caspase inhibitor, and DEVD is an inhibitor of ICE/CED-3 family of cysteine proteases.

Preparation of organotypic cultures

Fertilized eggs from White-Leghorn hens, purchased at a local farm,

were incubated at 38°C in a humidified atmosphere and were staged according to Hamburger and Hamilton (HH) criteria (Hamburger and Hamilton, 1951). Otic vesicles were dissected from chick embryos corresponding to HH18 (embryonic day 2.5) and cultured in serum-free M199 medium with Earle's salts (Biowhitaker, Walkersville, MD) supplemented with 2 mM glutamine (Biowhitaker) and antibiotics [50 IU/ml penicillin (Ern, Barcelona, Spain) and 50 μg/ml streptomycin (CEPA, Madrid, Spain)]. Treatments with 4 nM NGF, 1 nM IGF-I, 5 μM C_2-Cer, 5 μM dihydroceramide or 25 μM Cer-1-P were performed in the absence or presence of the different antibodies or inhibitors at 37°C in a water-saturated atmosphere containing 5% CO. C_2-Cer and manumycin A were prepared in DMSO and desipramine was prepared in methanol. The final concentration of DMSO or methanol in culture medium was 0.01%, which had no detectable effect on otic vesicle cultures. Otic vesicles cultured in medium without additions or, when indicated, with solvents, were taken as control values.

Western blot analysis

For western blotting, HH18 otic vesicles (1, 8 or 12 otic vesicles/datapoint for PCNA, PARP or Akt immunodetection, respectively) were made quiescent by incubation overnight in serum-free medium. Treatments were carried out at 37°C for 16 hours for PCNA and PARP analysis and 30 minutes for the Akt study. Afterwards, otic vesicles were homogenised in Laemmli Buffer (1.5×) with 1 mM phenylmethylsulfonyl-fluoride and frozen immediately. Gels were loaded with solutions containing equal amounts of proteins. Otic vesicle proteins were subjected to SDS-PAGE on 8% polyacrylamide gels for PARP or 12% gels for PCNA and Akt analysis. The electrophoresed proteins were transferred onto PVDF membranes using a Bio-Rad Trans Blot according to the manufacturer's instructions. After incubation with blocking solution (5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20), blots were probed with the appropriate specific primary antibodies for 2 hours at room temperature or overnight at 4°C. All antibodies were diluted in blocking solution except anti-phospho-Akt antibodies, which were diluted in Tris-buffered saline with 0.1% Tween 20 containing 5% bovine serum albumine. Blots were subsequently washed and then incubated with the adequate secondary antibodies conjugated with peroxidase for 1 hour at room temperature. The immunoblots were developed with a chemiluminiscence system (Dupont-NEN, Boston, MA) and exposed to X-ray film (Konica). Films were scanned in a Studio Scan II (Agfa) and the bands were quantified by densitometry with NIH Image 1.59 software.

Explant labelling and determination of ceramide levels

Otic vesicles were isolated from HH18 chick embryos and labelled with 25 μCi/ml of [^{3}H]palmitic acid for 24 hours in the presence of 1% fetal bovine serum. They were then washed with PBS, placed in serum-free medium and stimulated at 37°C with 4 nM NGF for 3 hours. Cellular lipids were extracted, and labelled ceramide was purified as reported (Frago et al., 1998). Briefly, lipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959), except that phases were separated by adding 2 M KCl in 0.2 M H_3PO_4 instead of water. The organic phase was dried under a N_2 stream and the lipid extracts were applied to thin-layer plates (silica Gel G60 t.l.c.), which were developed twice with chloroform:methanol:acetic acid (9:1:1, by volume). The material that co-migrated with cold ceramide standards was scraped and extracted with 1 ml methanol/H_2O (1:1 by volume). Radioactivity associated with the spots was determined by scintillation counting.

Assessment of apoptosis

Two techniques were used to study apoptotic cell death: TUNEL and DNA fragment ELISA. Distribution of apoptotic cells in the otic vesicle was determined by Tdt-mediated dUTP nick end labelling (TUNEL) of the fragmented DNA with the modifications reported previously (Blaschke et al., 1996) and adapted to whole organ labelling (Frago et al., 1998; Díaz et al., 1999). Briefly, otic vesicles were fixed overnight with 4% (w/v) paraformaldehyde in 0.1 M PBS pH 7.4 and permeated by four incubations with 1% (w/v) Triton X-100 in PBS for 30 minutes at room temperature and one incubation with 20 μg/ml proteinase K (Roche Molecular Biochemicals) for 10 minutes at 37°C. The otic vesicles were then incubated with the terminal deoxynucleotidyl-transferase buffer for 30 minutes at 37°C and subsequently incubated with 0.1 U/μl terminal deoxynucleotidyl-transferase and 10 μM biotin-16-deoxy-UTP (Roche Molecular Biochemicals) for 2.5 hours at 37°C. The reaction was stopped by incubation with 2 mM EDTA in PBS for 1 hour at 65°C. TUNEL labelling was visualized by incubation with Cy2-streptavidin (Amersham Pharmacia Biotech, Rainham, Essex, UK), which was followed by transparentation with 70% glycerol in PBS (v/v) and mounting for epifluorescence. Fluorescence was analysed in a MRC1024 BioRad confocal microscope. At least five otic vesicles per condition were assayed in three different experiments. In parallel, TUNEL assays on 25 μm cryostat sections of HH18 chicken embryos were performed essentially by following manufacturer's instructions (In situ Cell Death detection kit, POD, Roche Molecular Biochemicals), as described previously (Sanz et al., 1999a). Briefly, sections were fixed in 4% paraformaldehyde (w/v) for 20 minutes and then rinsed in 0.1% Triton X-100 with 0.1% sodium citrate for 2 minutes. Sections were then incubated with the terminal deoxynucleotidyl transferase enzyme in buffer containing fluorescein nucleotides for 1 hour at 37°C. The TUNEL signal was visualized with an antibody antifluorescein coupled to peroxidase for 30 minutes at 37°C. At least nine embryos were sampled in three different assays.

Otocyst cell death was quantified by using the Cell Death Detection ELISA (Roche Molecular Biochemicals), which was based on the detection of histone-associated DNA fragment in the cytoplasm of cells. Quiescent HH18 otic vesicles were cultured in serum-free medium with different additives for 16 hours and then processed for apoptosis determination. A single HH18 cultured otic vesicle was homogenised in 100 μl of the supplied incubation buffer and the cell extracts subjected to ELISA determination following basically the instructions of the supplier.

Whole mount in situ hybridization

In situ hybridisation was performed on chicken embryos as described previously (Wilkinson, 1992). A single-stranded chicken $p75^{NTR}$ RNA probe (A. Rodríguez-Tebar, Instituto Cajal, Madrid, Spain) was prepared by transcription of the linearized plasmid pKS-p75 with *Xba*I, using T3 RNA polymerase (Promega, Madison, WI) (Large et al., 1989). The control sense probe was prepared by using T7 RNA polymerase (Promega, Madison, WI) after linearizing pKS-p75 with *Hin*dIII. Embryos were incubated overnight at 70°C with 1 μg/ml digoxigenin-labelled RNA $p75^{NTR}$ probe in hybridization mix (50% formamide, 1.3×SSC pH 4.5, 5 mM EDTA, 50 μg/ml yeast RNA, 0.2% Tween 20, 0.5% CHAPS and 100 μg/ml heparin). To elicit colour, embryos were incubated with a 1/2000 dilution of anti-digoxigenin-alkaline phosphatase antibody (Roche Molecular Biochemicals). Colour was developed with 0.26 mg/ml nitroblue tetrazolium chloride and 0.175 mg/ml 5-bromo-4-chloro-3 indolyl-phosphate prepared in NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$ and 0.1% Tween 20). For histological examination, embryos were post-fixed in 4% paraformaldehyde, embedded in gelatine-albumin and sectioned with a vibratome (Leica VT 1000M, Heidelberg, Germany) at a thickness of 60 μm. At least 12 embryos were sampled in three different experiments.

Immunocytochemistry

Immunocytochemistry was performed on frozen sections obtained from HH18 chicken embryos fixed overnight in 4% paraformaldehyde (w/v), dehydrated in 30% sucrose and then included in Tissue Teck (Miles Diagnostics Div., Kankakee, IL). Frozen embryos were sectioned at a thickness of 20 μm with a cryostat (Leitz, Jena, Germany). Sections were placed on polylysine-coated slides and processed for $p75^{NTR}$ receptor analysis following the procedures previously described (Casaccia-Bonnefil et al., 1996; Sanz et al., 1999a). Briefly, sections were incubated in 3% H_2O_2 in methanol for 20 minutes and then blocked in a solution of 0.1% Triton X-100 in PBS containing 10% goat serum for 1 hour. Sections were left incubating overnight with rabbit polyclonal anti-$p75^{NTR}$ antibody (1/1000 in the blocking solution) in a humid chamber at 4°C. Afterwards sections were incubated in EnVision+ anti-rabbit peroxidase-conjugated secondary antibodies (Dako, Copenhagen, Denmark) for 30 minutes and peroxidase was developed with 0.5 mg/ml diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H_2O_2. At least 10 embryos were analyzed in three different experiments.

Results

IGF-I counteracts NGF-induced apoptosis in cultured otic vesicles

The dynamic balance between proliferation and programmed cell death during early otic development is carefully regulated (Alvarez and Navascués, 1990; Fekete et al., 1997; Sanz et al., 1999a). We have previously demonstrated that IGF-I promotes both proliferation and survival in the cultured otocyst, whereas NGF acts as an apoptosis-inducing factor (Frago et al., 1998; Sanz et al., 1999a). Therefore, we here explore the possible interactions between the pathways activated by IGF-I to prevent apoptosis and those activated by NGF to induce cell death.

The first step was to study the resulting effect when both factors were acting together on cultured otic vesicles. As shown in Fig. 1, NGF increased the number of TUNEL-positive cells, particularly at the ventromedial wall of the otic vesicle, where the CVG is emerging (Fig. 1, compare B with A) and within the CVG. C_2-Cer addition induced cell death with a less restricted pattern (Fig. 1C). IGF-I not only effectively reduced cell death caused by serum deprivation (Fig. 1, compare D with A), but it was also able to prevent apoptosis induced by NGF and C_2-Cer (Fig. 1E,F).

Expression of $p75^{NTR}$ in the developing inner ear

Once the epistatic effect of IGF-I on NGF was demonstrated, we characterized some of the pathways activated by NGF during cell death induction in the otic vesicles to later analyze the sites blocked by IGF-I. The presence of NGF receptors in the otic vesicle epithelium and CVG has been reported in several species (Hallbook et al., 1990; von Bartheld et al., 1991; Schecterson and Bothwell, 1994; Wu and Oh, 1996). We extended these reports by assessing the presence of $p75^{NTR}$ transcripts in HH18 chicken otic vesicles by whole mount in situ hybridization and immunohistochemistry (Fig. 2). The expression of $p75^{NTR}$ transcripts and protein was high in restricted areas of the otic vesicle epithelium and within the CVG (Fig. 2A,B). $p75^{NTR}$ expression was also high in the mesenchyme surrounding the otocyst. Furthermore, apoptotic cell death was detected in the same areas both in vivo and in cultured otic vesicles treated with NGF (Fig. 2C-E). Therefore, $p75^{NTR}$ was expressed within the areas of high cell death in the otocyst at the developmental stage studied.

$p75^{NTR}$ and caspase activation mediate NGF-induced apoptosis in the otic vesicle

The involvement of $p75^{NTR}$ in NGF pro-apoptotic actions was studied by using the 9651 anti-$p75^{NTR}$ antibody, which effectively blocks NGF binding to $p75^{NTR}$ (Huber and Chao, 1995). The levels of cytoplasm-soluble nucleosomes were measured in otic vesicle extracts to quantify cell death caused

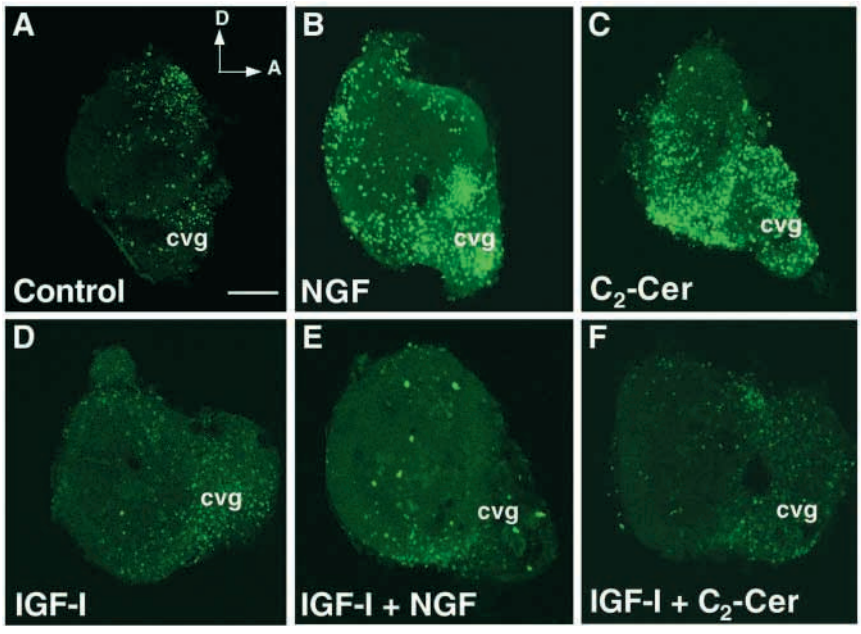

Fig. 1. IGF-I protects otic vesicles from NGF-induced cell death. Apoptotic cell death was visualised by TUNEL in cultured otic vesicles. Otic vesicles were isolated from HH18 chicken embryos, made quiescent and cultured for 8 hours in serum-free medium (A, control), 4 nM NGF (B), 5 μM C_2-Cer (C) or 1 nM IGF-I (D) or the combination of IGF-I with NGF (E) or C_2-Cer (F). Compiled projections of the whole otic vesicle are shown. All otic vesicles have the same orientation: D (dorsal), A (anterior), cvg (cochleovestibular ganglion). The images shown are representative of at least three different experiments, using five otic vesicles by condition. Bar, 100 μm.

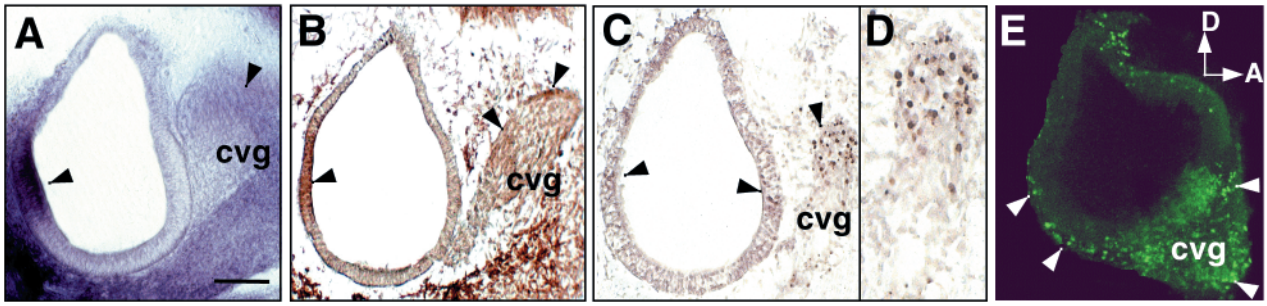

Fig. 2. p75^{NTR} is expressed in the developing inner ear. (A) p75^{NTR} mRNA expression was detected by whole mount in situ hybridization in HH18 chicken embryos. Arrowheads point to major patches of p75 expression, within the otic epithelium and in the CVG (cvg). The microphotograph shows a 60 μm parasagittal vibratome section. At least 12 embryos were sampled in three different experiments. (B) Immunolocalization of p75^{NTR} protein was positive in the otic epithelium and in the CVG (arrowheads). The microphotograph shows a 25 μm parasagittal section from an HH18 chicken embryo. At least 10 embryos were sampled in three different experiments. (C,D) Detection of apoptotic cells by TUNEL labelling in a 25 μm parasagittal cryostat section from an HH18 embryo. D shows a magnification of the CVG area. (E) 2.5 μm optic section of an otic vesicle cultured in the presence of 4 nM NGF. Apoptotic positive cells were visualised by TUNEL labelling. C and E arrowheads point to apoptotic cells in the otic epithelium and CVG. C and E microphotographs are representative of at least three different experiments performed in triplicate. A, anterior; D, dorsal; cvg marks the CVG area. Bar, 100 μm (A, B, C and E), 50 μm (D).

Table 1. NGF-induced cell death is mediated by p75^{NTR} in the otic vesicle

Control	100	(*n*=13)
NGF	149±5**	(*n*=13)
NGF + αNGF	118±6#	(*n*=5)
NGF + [9651]αp75	97±11#	(*n*=8)
NGF + [9992]αp75	133±5*	(*n*=8)
αNGF	107±13#	(*n*=6)
[9651]αp75	88±16#	(*n*=5)
[9992]αp75	98±6#	(*n*=8)

Otic vesicles were isolated from HH18 chicken embryos, made quiescent by an overnight incubation in serum-free medium and treated with NGF (4 nM), αNGF (2 μg/ml), [9651]αp75 (1:100) or the non-blocking antibody [9992]αp75 (1:100) for 16 hours. Soluble nucleosomes were quantified in single otic vesicle explants. For each experiment the absorbance obtained in the absence of serum (control) was given a value of 100. *n* is the total number of explants tested in at least three different experiments. Values are expressed as mean±s.e.m. Statistical significance estimated by ANOVA was as follows: $^{**}P<0.005$ versus control, $^{*}P<0.05$ versus control and $\#P<0.005$ versus NGF.

Table 2. Involvement of caspases in p75^{NTR} and C_2-Cer-dependent cell death

Control	100	(*n*=17)
NGF	149±5*	(*n*=13)
NGF + zVAD	80±12#	(*n*=8)
NGF + DEVD	101±6#	(*n*=16)
C_2-Cer	176±7*	(*n*=19)
C_2-Cer + zVAD	116±4§	(*n*=10)
C_2-Cer + DEVD	122±7§	(*n*=13)
Dihydroceramide	109±1	(*n*=3)
ZVAD	80±12	(*n*=9)
DEVD	94±21	(*n*=11)

Quiescent HH18 otic vesicles were treated for 16 hours with NGF (4 nM), C_2-Cer (5 μM) or dihydroceramide (5 μM) alone or in combination with either zVAD (25 μM) or DEVD (75 μM) as indicated. To quantify cell death soluble nucleosomes were measured as described in the Materials and Methods section. For each experiment the absorbance obtained in the absence of serum (Control) was given a value of 100. *n* is the total number of explants tested in at least three different experiments. Values are presented as mean±s.e.m. Statistical significance estimated by ANOVA was as follows: $^{*}P<0.005$ versus Control, $\#P<0.005$ versus NGF and $^{\S}P<0.005$ versus C_2-Cer.

by treatment with NGF or NGF plus anti-p75^{NTR} antibodies. Table 1 shows that apoptosis induced by NGF was completely prevented by the 9651 anti-p75^{NTR} antibody but not by treatment with the control non-blocking 9992 anti-p75^{NTR} antibody. These results indicate that in the otic vesicle the apoptotic response to NGF requires binding to p75^{NTR}. In addition, the specificity of NGF actions was confirmed by culturing the otic vesicle explants in the presence of NGF plus anti-NGF antibody. Under these conditions, NGF did not induce cell death. Treatment of otic vesicles with any of these antibodies alone had no appreciable effect (Table 1). Therefore, NGF effects on otic vesicle apoptosis are specific for this neurotrophic factor and are mediated through binding to p75^{NTR}.

To evaluate the implication of caspases in p75^{NTR}-dependent NGF-induced cell death, otic vesicles were cultured in the presence of caspase inhibitors, which completely suppressed death induced by NGF (Table 2). Treatment of otic vesicles with either zVAD (25 μM) or DEVD (75 μM) completely blocked NGF-induced apoptosis, whereas the addition of zVAD or DEVD had no effect on basal cell death. Caspase inhibitors also prevented C_2-Cer-induced cell death. The specificity of C_2-Cer actions was tested using dihydroceramide, an inactive synthetic analogue of C_2-Cer. The addition of 5 μM dihydroceramide had no effect on the relative amount of oligonucleosomes in the cytoplasm, indicating that C_2-Cer actions were specific (Table 2).

TUNEL staining of cultured otic vesicles confirmed these results (Fig. 3). Otic vesicles cultured in serum-free medium presented basal levels of cell death, whereas NGF treatment increased the number of TUNEL-positive cells (Fig. 3A,B). By contrast, pre-treatment with either anti-NGF antibodies or anti-p75^{NTR} blocking antibodies led to a significant reduction in cell death when compared with otic vesicles cultured with NGF alone (Fig. 3B-D). Pre-treatment of the otic vesicles with caspase inhibitors also blocked NGF-induced apoptosis in the otic vesicle epithelium and the CVG (Fig. 3F,H). Treatment

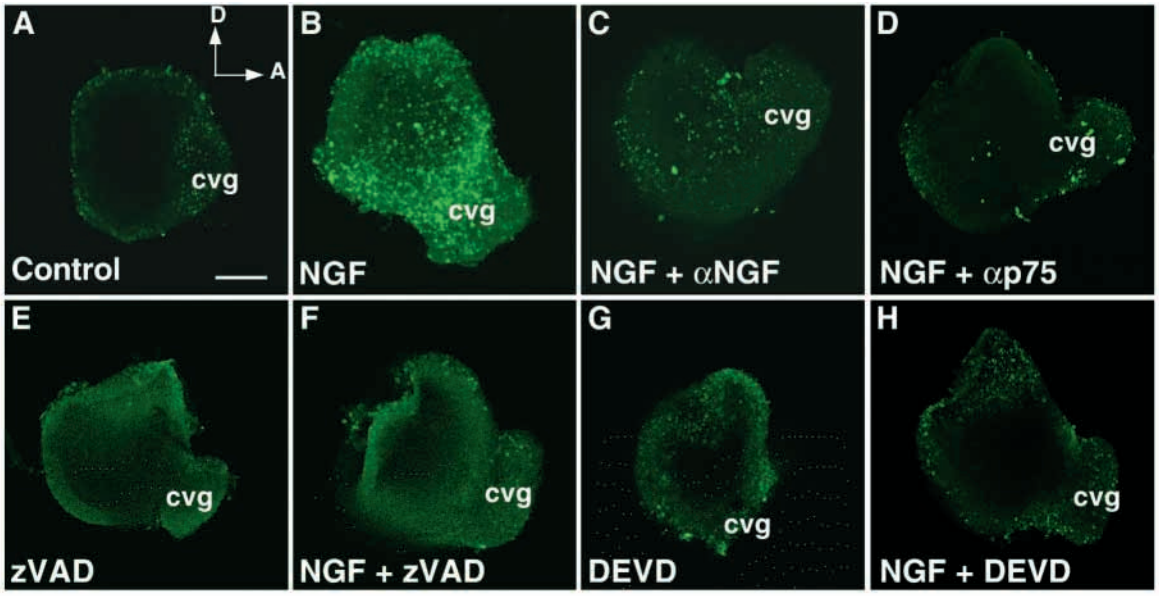

Fig. 3. p75^{NTR} and caspases are involved in NGF-induced cell death in the otic vesicle. Apoptotic cell death was visualised by TUNEL. Otic vesicles were isolated from HH18 chicken embryos, made quiescent and cultured for a further period of 8 hours in serum-free medium (A, Control) or 4 nM NGF (B) alone or in combination with 2 μg/ml anti-NGF antibody (C), (1:100) 9651 anti p75^{NTR} antibody (D) or caspase inhibitors, 25 μM zVAD (F) and 75 μM DEVD (H), as indicated in the panels. Explants were pre-treated for 30 minutes with the antibodies or caspase inhibitors. All the otic vesicles have the same orientation: D (dorsal); A (anterior); cvg (cochleovestibular ganglion). Compiled projections of the whole otic vesicle are shown. Representative microphotographs of three independent experiments using five otic vesicles by condition are shown. Bar, 100 μm.

with either the antibodies or the inhibitors alone had no effect (data not shown and Fig. 3E,G, respectively).

To further explore NGF-p75^{NTR} downstream signalling, intracellular ceramide levels and PARP cleavage were determined in the absence or presence of anti-p75^{NTR} antibodies, anti-NGF antibodies or caspase inhibitors (Fig. 4). Treatment with either anti-NGF or 9651 anti-p75^{NTR} antibodies impaired NGF induction of ceramide accumulation (Fig. 4A). The next step was to study the roles of the caspase subfamilies in the generation of endogenous ceramide. Fig. 4A shows that zVAD, but not DEVD, was able to impair the increase in ceramide levels caused by NGF. Therefore, these data suggest that the increase in the intracellular ceramide content produced by NGF is secondary to the activation of a subset of zVAD-sensitive DEVD-insensitive caspases, most probably initiator caspases. The degree of PARP cleavage to the 85 kDa PARP fragment, a marker of caspase-3-dependent apoptosis (Lazebnik et al., 1994), was also determined in otic vesicles cultured under different conditions. NGF increased the proteolytic cleavage of PARP by 2.6-fold, which was prevented by pre-treatment with either blocking anti-p75^{NTR} antibodies or caspase inhibitors (Fig. 4B). PARP cleavage was also increased in otic vesicles cultured in the presence of C_2-Cer by 4.5-fold, and this was again prevented by treatment with caspase inhibitors (Fig. 4C). Taken together these results suggest that following NGF binding to p75^{NTR}, initiator caspases are activated. This activation will lead to an increase in intracellular ceramide content that in turn will stimulate effector caspase-3-like proteases, leading to PARP degradation, DNA fragmentation and cell death.

NGF-induced cell death is dependent on ceramide generation

Next we examined the intracellular source of the ceramide released by the action of NGF (Fig. 4A). FB1 (50 μM), an inhibitor of de novo ceramide synthesis, reduced the NGF-dependent increase in ceramide levels by 50% and was also able to impair NGF-dependent cell death (Table 3). Desipramine (10 μM), an acid sphingomyelinase inhibitor, was also able to impair NGF actions by decreasing ceramide levels by 52% and blocking cell death (Table 3). On the other hand, the neutral sphingomyelinase inhibitor manumycin A did not cause any effect at the doses tested (10 to 100 μM, data not shown).

IGF-I activates Akt and blocks NGF-induced ceramide increase

Finally, we explored the crosstalk between the IGF-I and NGF intracellular pathways. Treatment of cultured otic vesicles with IGF-I prevented NGF-induced ceramide accumulation (Fig. 4A; Fig. 5A). Ceramide is phosphorylated in vivo by a ceramide kinase to give Cer-1-P (Dressler and Kolesnick, 1990), which is a cytoprotector for the otocyst (Frago et al., 1998). In vitro, bacterial DGK carries out the same reaction (Schneider and Kennedy, 1973). Ceramide kinase has been recently cloned, purified and its biochemical activity characterized in vitro (Sugiura et al., 2002). Ceramide kinase has a DGK catalytic domain (Sugiura et al., 2002), which is the target of the DGK inhibitor R59949 (Jiang et al., 2000). To test whether IGF-I is further controlling intracellular ceramide levels by modulating ceramide phosphorylation, otic vesicle

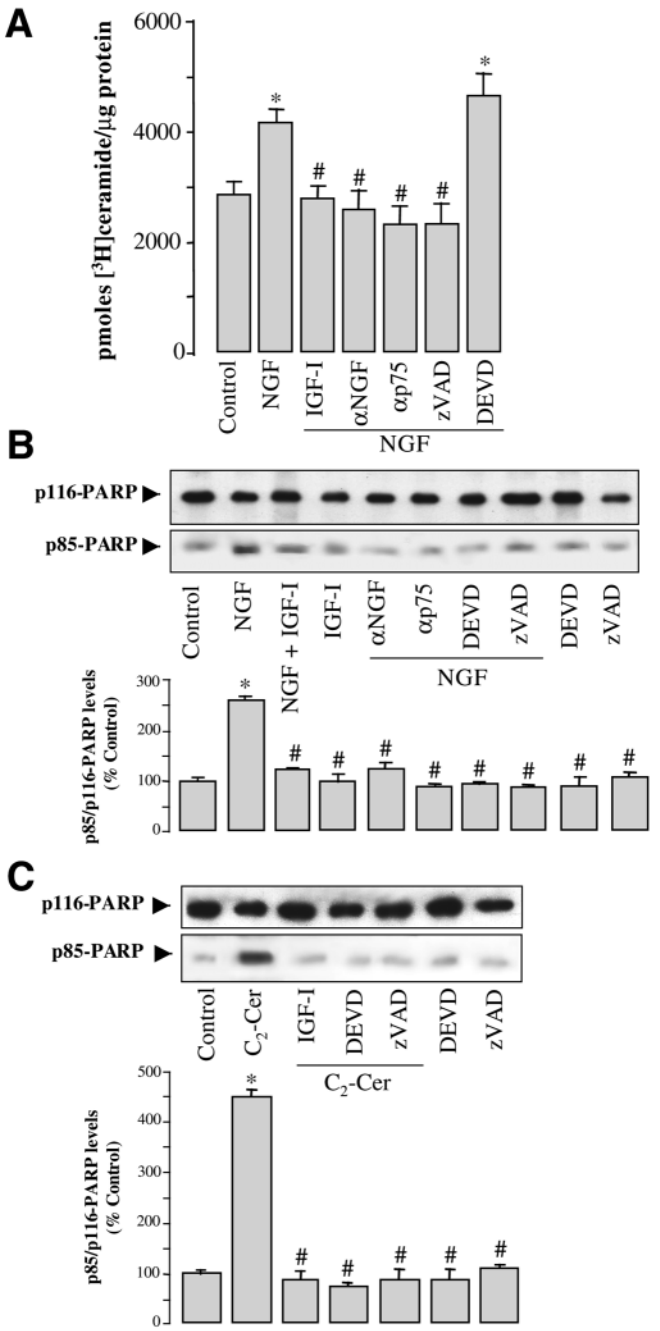

Fig. 4. Modulation of NGF-induced ceramide accumulation and PARP hydrolysis. (A) Modulation of ceramide accumulation by NGF. Five HH18 otic vesicles/per datapoint were labelled with 25 μCi/ml of [^{3}H]palmitic acid for 24 hours as described in Materials and Methods. Otic vesicles were then carefully washed and incubated for 3 hours without additives (control) or with 4 nM NGF alone or in combination with 1 nM IGF-I, 2 μg/ml anti-NGF antibody, 9651 anti-p75^{NTR} antibody (1:100), 25 μM zVAD or 75 μM DEVD. Ceramide accumulation was measured as described in Materials and Methods. Results are expressed as mean±s.e.m. of at least five independent experiments performed in triplicate. Statistical significance estimated by ANOVA was as follows **P*<0.005 versus control, #*P*<0.005 versus NGF. (B) NGF-induced cell death triggers PARP-hydrolysis. Hydrolysis of native poly-ADP-ribose polymerase (p116-PARP) produces a fragment of 85 kDa (p85-PARP). Quiescent HH18 otic vesicles (8 otic vesicles per data point) were incubated for 16 hours either in serum-free medium (Control) or in the presence of 4 nM NGF alone or in combination with 1 nM IGF-I, 2 μg/ml anti-NGF antibody, 9651 (1:100) anti-p75^{NTR} antibody, 25 μM zVAD or 75 μM DEVD. Incubation with the non-blocking p75^{NTR} antibody or agonist vehicles had no effect on PARP induction (data not shown). Otic vesicle lysates were analysed by western blotting with an anti-PARP antibody that recognizes both the intact and the proteolysis fragment. A representative blot of one of three independent experiments is shown. Densitometric quantification is shown as bars. Statistical significance estimated by ANOVA was as follows **P*<0.005 versus control, #*P*<0.005 versus NGF. (C) C_2-Cer-induced cell death also triggers PARP-hydrolysis. Otic vesicles were incubated in serum-free medium (Control) or 5 μM C_2-Cer alone or in combination with 1 nM IGF-I, 25 μM zVAD or 75 μM DEVD. Otic vesicle lysates were analysed by western blotting with an anti-PARP antibody as above. A representative blot of one of three independent experiments is shown. Densitometric quantification is shown as bars. Statistical significance estimated by ANOVA was as follows **P*<0.005 versus control, #*P*<0.005 versus C_2-Cer.

cultures were treated with R59949, which produced a slight increase in basal apoptotic cell death but was unable to affect IGF-I actions (Table 4). The cytoprotective effect of Cer-1-P is shown for comparison (Table 4). In the presence of either NGF or C_2-Cer, R59949 effectively impaired the survival actions of IGF-I. Moreover, treatment with R59949 blocked the actions of IGF-I on NGF-induced increase in ceramide levels (Fig. 5A) and on the activation of JNK (data not shown). IGF-I prevents JNK activation induced by both serum deprivation and NGF (Sanz et al., 1999a). These results suggest that the protective effect of IGF-I on cell death may be mediated by inducing ceramide phosphorylation.

Activation of the pathway initiated by phosphorylation of the Akt serine/threonine kinase by IGF-I is central to cell survival (Dudek et al., 1997; Zheng et al., 2000). In cultured otic vesicles, IGF-I effectively stimulated the phosphorylation of Akt at Ser473 (4.7-fold) and Thr308 (4-fold) residues (Fig. 5B and data not shown, respectively). NGF and C_2-Cer did not induce changes in the levels of Akt phosphorylation and were unable to alter the induction by IGF-I of the phosphorylation at Ser473-Akt (4.1- and 3.5-fold, respectively) and Thr308 (3.8- and 2.3-fold, respectively) (Fig. 5B and data not shown). However, pre-treatment with R59949 plus NGF or C_2-Cer decreased IGF-I-induced Ser473- and Thr308-Akt phosphorylation (2.8- and 2.2-fold, respectively for Ser473-Akt phosphorylation; 2.2- and 1.2-fold for Thr308-Akt phosphorylation), whereas in the absence of IGF-I R59949 alone or in combination with NGF had no effect (data not shown). Treatment with Cer-1-P also increased Akt phosphorylation levels by 2.7- and 1.9- fold at Ser473 and Thr308 residues, respectively. On the other hand, phosphatidic acid (10-50 μM), the natural product of DGK, had no effect on Akt phosphorylation (25 μM, 0.9±0.2 fold) at the same time points. Changes in the degree of phosphorylation of Akt were paralleled by changes in the levels of PCNA, a marker of cell proliferation (Fig. 5B).

Discussion

In summary, here we show that: (i) p75^{NTR} is expressed in the

Table 3. NGF-induced cell death is ceramide generation dependent

	pmoles [^{3}H]ceramide/μg protein		Cell death	
Control	3640±216	(*n*=6)	100	(*n*=6)
NGF	5522±355*	(*n*=6)	154±9*	(*n*=6)
NGF + FB1	4576±350	(*n*=6)	115±4##	(*n*=7)
NGF + Desipramine	4556±335	(*n*=6)	109±15#	(*n*=6)
FB1	3053±622	(*n*=3)	90±6	(*n*=3)
Desipramine	3680±11	(*n*=3)	101±18	(*n*=4)

Otic vesicles were dissected from HH18 chick embryos. For the cell death assays otic vesicles were deprived of serum and cultured for 16 hours with NGF (4 nM) or NGF plus 50 μM fumonisin B1 (FB1) or 10 μM desipramine. One hour of pre-treatment was performed in those conditions where inhibitors were used. Quantification of nucleosomes was performed in single otic vesicle explants and was normalized versus the control. *n* is the total number of explants tested in at least two different experiments. Ceramide generation was evaluated in parallel experiments as described in Materials and Methods. Values are expressed as mean±s.e.m; *n* represents the total data point number with five otic vesicle explants per point. Statistical significance estimated by ANOVA was as follows: **P*<0.005 versus control, ##*P*<0.005 versus NGF, #*P*<0.05 versus NGF.

otic vesicle epithelium and CVG; (ii) NGF-induced apoptosis in the otic vesicle is p75^{NTR} dependent, since cell death can be prevented by blocking the p75^{NTR} extracellular domain; (iii) NGF induces ceramide generation by activating both de novo synthesis and acid sphingomyelinase activity; (iv) initiator and effector caspases are involved in NGF-induced cell death; and (v) the protective role of IGF-I on NGF-induced cell death is accompanied by a decrease in the intracellular levels of ceramide and inducing phosphorylation of the Akt kinase.

Cell death, which normally takes the route of apoptosis, is a physiological process during development and morphogenesis (Raff et al., 1993). Apoptosis can be induced or prevented by a variety of stimuli that activate different intracellular signalling pathways, which converge to activate or inhibit a common pool of executioner molecules. Analysis of many linear intracellular signal transduction and apoptotic pathways has been the object of intense research. But how and where they intersect and whether this crosstalk results in synergy or antagonism is not known in most cellular contexts. Therefore, determining the sequence of events and the interdependencies involved in apoptosis signalling is an area of active research.

Among the neuronal pro-apoptotic factors, considerable attention is presently focused on NGF and its low-affinity receptor, p75^{NTR}. It has been proposed that the relative levels of high- and low-affinity NGF receptors determine cell fate (Lee et al., 2001; Chao and Bothwell, 2002). The cellular response to NGF will, therefore, depend on the developmental modulation of NGF receptors expression. In the present study, we show that in the otic vesicle NGF-induced apoptosis is mediated by binding to p75^{NTR}. A similar situation has been reported for embryonic retinal cells and postnatal oligodendrocytes where activation of p75^{NTR} increases cellular apoptosis (Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Frade, 2000; González-Hoyuela et al., 2001). In the otocyst, after binding to p75^{NTR}, NGF increases ceramide levels, and this increase can be reduced by treatment with inhibitors of acid sphingomyelinase and de novo synthesis. p75^{NTR}-induced apoptosis has been shown to trigger ceramide generation in different neural cell types including oligodendrocytes and neuroblastoma cells (Dobrowsky et al., 1994; Dobrowsky et al., 1995; Casaccia-Bonnefil et al., 1996; Lievremont et al., 1999). Although earlier studies pointed to a membrane-neutral sphingomyelinase associated with p75^{NTR} as the enzyme responsible for ceramide increase (Brann et al., 1999; Dobrowsky and Carter, 2000), such an enzyme has not yet been characterized and our results indicate that, in the otic vesicle, NGF actions are dependent on both the full activity of an acid sphingomyelinase and on an increase in de novo ceramide synthesis. These results are in agreement with the sustained increase in ceramide levels (up to 4 hours) induced by NGF in this system (Frago et al., 1998). Acid sphingomyelinase participates in stress-activated and developmental apoptosis (Peña et al., 1997; Morita et al., 2000; Cutler and Mattson, 2001) and p75^{NTR} overexpression induces cell survival in human Niemann-Pick fibroblasts, which lack acid sphingomyelinase activity (Roux et al., 2001). On the other hand, de-novo-synthesized ceramide has been reported to be crucial for apoptosis in different cellular settings including during early neural differentiation (Herget et al., 2000; Gomez del Pulgar et al., 2002).

Several studies indicate that ceramide generation is essential for apoptosis, but there are discrepancies concerning whether ceramide generation is upstream or downstream of caspases activation (Chinnaiyan et al., 1996; Mizushima et al., 1996; Hartfield et al., 1998). Some groups studying secondary cultures of adult cells have concluded that ceramide action is downstream of the initiator caspases but upstream from the executioner caspases, such as caspase-3 (Sweeney et al., 1998; Grullich et al., 2000; Craighead et al., 2000). Caspase-3 is thought to be primarily required for the nuclear changes that occur during apoptosis (Oppenheim et al., 2001). Furthermore, recent studies show that ceramide induces non-apoptotic programmed cell death with necrotic-like morphology (Mochizuki et al., 2002). We have studied NGF-induced ceramide generation in the presence of caspase inhibitors in organotypic cultures of the developing otocyst. We show that after NGF binding to p75^{NTR} only zVAD was capable of blocking NGF-induced ceramide release. On the other hand, PARP-cleavage and cell death were impaired by both zVAD and DEVD, suggesting that the activation of both initiator and caspase-3-like proteases is required for PARP hydrolysis and cell death. Therefore, the activation of the caspase-3 occurs downstream of the increase in intracellular ceramide content produced in response to NGF. These data suggest that an increase in ceramide could be a secondary signal activated by caspases and capable of further activating these proteases. The ceramide increase may, therefore, be a reinforcement loop to commit cells to apoptosis.

In the otic vesicle, the survival actions of IGF-I are independent from the stimuli responsible for the initiation of

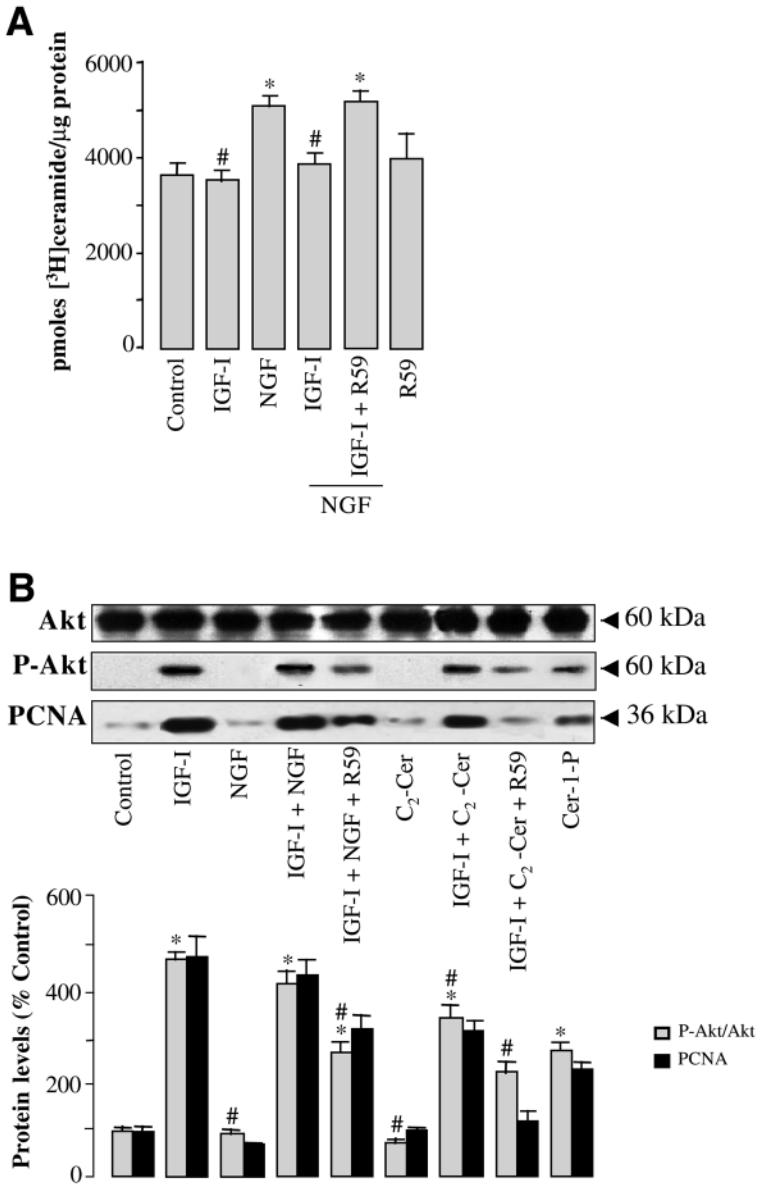

Fig. 5. IGF-I increases Akt phosphorylation and PCNA levels. (A) Modulation of NGF-induced ceramide accumulation by R59949. Ceramide accumulation was measured as described in Materials and Methods. Otic vesicles were incubated for 3 hours without additives (Control), 1 nM IGF-I, 1 μM R59949 or with 4 nM NGF alone or in combination with IGF-I and R59949. Statistical significance estimated by ANOVA was as follows **P*<0.005 versus control, #*P*<0.005 versus NGF. (B) Quiescent HH18 otic vesicles were incubated for 30 minutes with 1 nM IGF-I, 4 nM NGF, 5 μM C_2-Cer, 25 μM Cer-1-P or combinations of these factors for the determination of Akt or for 24 hours to measure PCNA levels. When indicated, a 30 minute pre-treatment with 1 μM R59949 was performed. Otic vesicle lysates were analysed by western blotting for total Akt levels, for the active phosphorylated form of Akt (Ser473 P-Akt) and for PCNA levels. Representative blots are shown. The lower panel shows densitometric quantification values of activated Akt (Akt-P) referred to total Akt levels (grey bars) and of PCNA (dark bars). Control cultures were taken as 100. Values are given as mean±s.e.m. of independent experiments performed in duplicate. The statistical significance between incubations under different conditions estimated by ANOVA was as follows: **P*<0.005 versus control, #*P*<0.001 versus IGF-I. R59949 plus IGF-I and NGF or C_2-Cer were statistically significant (*P*<0.001) with respect to their equivalent R59949-untreated conditions.

apoptosis, protecting it from serum withdrawal, NGF and C_2-Cer-induced cell death. *Igf-1*-knockout mice show increased apoptosis and activated caspase-3 in the cochlear ganglia (Camarero et al., 2001). By contrast, transgenic mice that overexpress IGF-I present decreased levels of activated caspase-3 and a general impairment in apoptotic pathways (Chrysis et al., 2001). In parallel to these studies, it is known that a major pathway for the anti-apoptotic actions of IGF-I is the activation of the Akt kinase (Dudek et al., 1997). Phosphorylation of Akt at threonine 308 by phosphoinositide-dependent kinase 1 is followed by autophosphorylation at serine 473 to produce an activated Akt whose role is to facilitate survival by phosphorylation of downstream substrates (Brazil and Hemmings, 2001). Akt kinase plays a crucial role in supporting NGF-dependent survival through Trk receptors, whereas activation of the $p75^{NTR}$ receptor can initiate a cell death cascade in neurons and glial cells (Kaplan and Miller, 2000). Here we show that IGF-I acts at different levels to protect otic vesicles from NGF-induced cell death. First, IGF-I blocks the intracellular increase in ceramide generation elicited by NGF and second, IGF-I activates Akt. Therefore, the protective role of IGF-I in $p75^{NTR}$-dependent cell death is, at least in part, mediated by decreasing ceramide levels with a concomitant activation of Akt phosphorylation. Cer-1-P, a molecule that is reported to be a cytoprotector for otic vesicle explants, also activated Akt. The crosstalk between PI 3-kinase, an enzyme upstream in the intracellular cascade of Akt, and the sphingomyelinase pathways has been shown to be important for the balance between cell survival and death decisions (Burow et al., 2000). Indeed, PI 3-kinase downregulation by stress is dependent on acid sphingomyelinase (Zundel and Giaccia, 1998). C_2-Cer has been reported to inhibit Akt activity (Schubert et al., 2000; Teruel et al., 2001); however, in the otic vesicle, IGF-I is able to protect the otocyst from C_2-Cer-induced apoptosis, suggesting that in addition to Akt activation there is another mechanism(s) by which IGF-I blocks cell death induced by exogenous C_2-Cer. One possible fate for ceramide is conversion to Cer-1-P by ceramide kinase, which has a DGK catalytic domain (Sugiura et al., 2002). By using R59949 we have explored the possibility that the protective effects of IGF-I on otic vesicle survival could be due to an increase in Cer-1-P. In the presence of R59949, the protective action of IGF-I against apoptosis induced by NGF or C_2-Cer was lost, and this is associated with an increase in NGF-induced endogenous ceramide levels. Other lipid mediators such as phosphatidic acid or sphingosine-1-phosphate have no significant survival actions in this system as shown here and previously (Frago et al., 1998). These data suggest that IGF-I enhances the conversion of the pro-apoptotic ceramide to its phosphorylated cytoprotective metabolite Cer-1-P; therefore, the relative levels of ceramide and Cer-1-P could be an intracellular indicator for cell death/survival decisions.

Our results indicate that during the early development of the chicken inner ear the signalling pathways induced

Table 4. IGF-I protective effects on cell death induced by NGF and C2-Cer

Control	100	(*n*=9)
IGF-I	84±5*	(*n*=9)
R59949	124±5*	(*n*=8)
IGF-I + R59949	89±6*	(*n*=8)
NGF	159±12*	(*n*=6)
NGF + IGF-I	106±3#	(*n*=8)
NGF + IGF-I + R59949	154±7*	(*n*=7)
C_2- Cer	162±17*	(*n*=6)
C_2- Cer + IGF-I	118±10§	(*n*=6)
C_2- Cer + IGF-I + R59949	155±11*	(*n*=6)
Cer-1-P	81±5*	(*n*=3)
Cer-1-P + NGF	143±13*	(*n*=8)

Quiescent otic vesicles were dissected from HH18 chick embryos and cultured for 16 hours with R59949 (1 μM), IGF-I (1 nM), NGF (4 nM), C_2-Cer (5 μM) or Cer-1-P (25 μM). A pretreatment of 30 minutes was done in those conditions where R59949 was used. Quantification of nucleosomes was made in single otic vesicle explants and normalized versus control value taken as 100. *n* is the total number of explants tested in at least three different experiments. Values are presented as mean±s.e.m. Statistical significance estimated by ANOVA was as follows: ∗P<0.005 versus control, #P<0.005 versus NGF and §P<0.05 versus C_2-Cer.

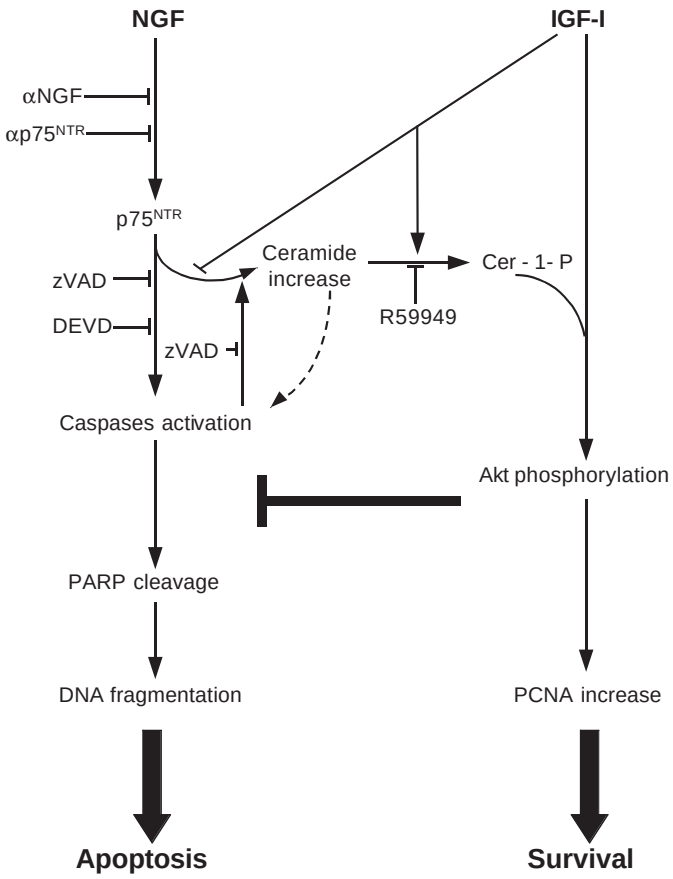

Fig. 6. NGF signalling through p75 in the otic vesicle. A proposed scheme showing the intracellular signalling pathways activated by NGF and IGF-I and how they balance cell death. Arrows denote facilitating action whereas crossbars indicate inhibitory influence.

by NGF and IGF-I form a complex network that regulates apoptotic cell death, a likely scenario for the regulation of early neural cell death (Fig. 6). We provide evidence that the molecular basis for the restricted apoptotic response to NGF in the otocyst involves modulation of the activation of death $p75^{NTR}$ versus survival receptors. Activation of survival signalling pathways implies blockage of the apoptotic pathways at several levels. Hence, some aspects of the regulation of inner ear development appear to occur thanks to the crosstalk between NGF and IGF-I signalling. We propose that the dynamic balance between levels of ceramide metabolites and the consequent regulation of Akt phosphorylation are important factors that determine whether a cell survives or dies.

This work was supported in part by grants from Dirección General de Investigación, Ciencia y Tecnología (PM99-0111) to I. V-N and (PM99-0094) to E. J. de la R. L.M.F. was supported by Europharma (Boehringer Ingelheim Inc.), Y.L. held a CSIC research contract and S.C. was supported by the Comunidad de Madrid. We are grateful to Julie A. Chowen, Ana Cuadrado, Flora de Pablo, David Jones, Isabel Merida and Agustin Rodríguez for helpful comments on this manuscript.

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