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Proinsulin/insulin is synthesized locally and prevents caspase- and cathepsin-mediated cell death in the embryonic mouse retina

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Abstract

Programmed cell death is an essential, highly regulated process in neural development. Although the role of insulin-like growth factor I in supporting the survival of neural cells has been well characterized, studies on proinsulin/insulin are scarce. Here, we characterize proinsulin/insulin effects on cell death in embryonic day 15.5 mouse retina. Both proinsulin mRNA and proinsulin/insulin immunoreactivity were found in the developing retina. Organotypic embryonic day 15.5 retinas cultured under growth factor deprivation showed an increase in cell death that was reversed by proinsulin, insulin and insulin-like growth factor I, with similar median effective concentration values via phosphatidylinositol-3-kinase activation. Although insulin and insulin-like growth factor I provoked a sustained Akt phosphorylation, proinsulin-induced phosphorylation of Akt was not found. Analysis of the growth factor deprivation-induced cell death mechanisms, using caspase and cathepsin inhibitors, demonstrated that both protease families were required for the effective execution of cell death. The insulin survival effect, which decreased the extent and distribution of cell death to levels similar to those found *in vivo*, was not enhanced by simultaneous treatment with caspase and cathepsin inhibitors, suggesting that insulin interferes with these protease pathways in the embryonic mouse retina. The mechanisms characterized in this study provide new details on early neural cell death and its genuine regulation by insulin/proinsulin.

Keywords: Akt, apoptosis, insulin-like growth factor I, neurogenesis, phosphatidylinositol-3-kinase, programmed cell death.

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During neural development, neuronal and glial cell populations are selected by cell death, which therefore contributes to the generation of the elaborate cytoarchitecture and connectivity of the nervous system (Kuan *et al*. 2000; Roth and D'Sa 2001; Yeo and Gautier 2004). The impact of cell death affecting neural precursors and immature neurons in the early stages of neural development, and its regulation by local factors, have only been partially defined (de la Rosa and de Pablo 2000; Boya and de la Rosa 2005).

In vitro approaches using transfected cell lines have elucidated several regulatory mechanisms of programmed cell death, although analysis in primary systems has been limited (Strasser *et al*. 2000; Joza *et al*. 2002). The vertebrate neuroretina, a part of the central nervous system, provides a model system to study the regulation of cell death, recognized as apoptosis, in a physiological context. Of the defined developmental periods of apoptosis in the vertebrate retina, the best characterized is a late phase in the second half of retinal development, which coincides with neuronal connectivity and synaptogenesis (Glücksmann 1940; Young 1984; Provis and van Driel 1985; Maslim *et al*. 1997; Marin-Teva *et al*. 1999). An earlier, less well-characterized phase of programmed cell death takes place in the first half of retinal development (Penfold and Provis 1986; Hensey and Gautier 1998; Diaz *et al*. 1999, 2000; Laemle *et al*. 1999; Biehlmaier *et al*. 2001).

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Abbreviations used: BrdU, bromodeoxyuridine; E, day of embryonic development; EC_{50}, median effective concentration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF, insulin-like growth factor; IR, insulin receptor; PI3K, phosphatidylinositol-3-kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.

The insulin-related growth factors, proinsulin, insulin and insulin-like growth factor (IGF) I and II, regulate multiple processes in neural cells, including survival during development and in adult life (de Pablo *et al*. 1993; de Pablo and de la Rosa 1995; Anlar *et al*. 1999; Lackey *et al*. 2000; Varela-Nieto *et al*. 2003). Although IGF-I is broadly accepted as a neurotrophic/neuroprotective factor, much less attention has been paid to other members of the insulin family, specifically insulin and its precursor, proinsulin. Receptors for insulin and IGF-I are expressed in the developing retina in several vertebrates (Valentino *et al*. 1990; de la Rosa *et al*. 1994; Calvaruso *et al*. 1996; Funkenstein *et al*. 1997; Van Kleffens *et al*. 1999), however, supporting a possible role of both factors in retinal development.

We have shown previously that, prior to IGF-I expression, proinsulin and insulin act as survival factors during early neural development in the chick, possibly signalling through a low-discriminating, hybrid insulin/IGF-I receptor (Morales *et al*. 1997; Diaz *et al*. 1999, 2000; Garcia de Lacoba *et al*. 1999; Hernandez-Sanchez *et al*. 2002, 2003). Here, we characterize several proinsulin/insulin-modulated pathways in the regulation of early neural cell death in the embryonic mouse retina.

Materials and methods

Materials

Hyaluronidase type IV, collagenase, trypsin, bromodeoxyuridine (BrdU), EDTA, anti-β-tubulin and anti-BrdU antibodies were purchased from Sigma (St Louis, MO, USA). Recombinant human proinsulin and insulin were obtained from Sigma, and recombinant human IGF-I was purchased from Preprotech (Rocky Hill, NJ, USA). LY294002, Boc-D-fmk, z-VEID-fmk, z-YVAD-fmk, z-FA-fmk and z-FF-fmk were purchased from Calbiochem (San Diego, CA, USA) and z-DEVD-fmk was obtained from R & D Systems (Minneapolis, MN, USA). The apoptosis detection system and anti-activated caspase 3 antibody were obtained from Promega (Madison, WI, USA). 4′,6-Diamidino-2-phenylindole and Texas Red-conjugated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). Rabbit polyclonal anti-phospho-Akt (Ser473) was obtained from Cell Signaling (Beverly, MA, USA). Goat polyclonal anti-Akt1 was purchased from Santa Cruz (Santa Cruz, CA, USA), anti-βIII-tubulin antibody (TUJ1) was obtained from Berkeley Antibodies (Berkeley, CA, USA), anti-rabbit Alexa 488-conjugated secondary antibody was purchased from Molecular Probes (Carlsbad, CA, USA) and the appropriate peroxidase-conjugated secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA, USA). Super Signal West Pico chemiluminescent substrate was obtained from Pierce (Rockford, IL, USA). Trizol reagent, deoxyribonuclease and the SuperScript first-strand synthesis system for RT-PCR were purchased from Invitrogen (Carlsbad, CA, USA). Taq DNA polymerase and deoxynucleotide triphosphates were obtained from Roche Diagnostics (Mannheim, Germany). The ultrasensitive mouse insulin ELISA was obtained from Mercodia (Uppsala, Sweden).

Mouse embryo

C57BL/6 mouse embryos were obtained from pregnant mice reared in a 12-h light : 12-h dark cycle at 20°C. Mice were mated and the morning of the appearance of a vaginal plug was designated as embryonic day 0.5 (E0.5). Mouse embryos were collected from pregnant females killed on day E15.5 according to European Union Guidelines for animal care.

Neuroretina organotypic culture

After removal of the eyes, the retinas were dissected free of other tissues and pre-incubated in a chemically defined medium (Diaz *et al*. 1999) supplemented with 0.5 mg/mL hyaluronidase type IV for 15 min at 37°C in a 5% CO_2 atmosphere. Retinas were then transferred to fresh basal medium and cultured for 6 h. Where indicated, recombinant human proinsulin, insulin or IGF-I (at the indicated doses), LY294002 (50 μM), Boc-D-fmk (38 μM), z-DEVD-fmk (150 μM), z-VEID-fmk (38.7 μM), z-YVAD-fmk (150 μM), z-FA-fmk (150 μM), z-FF-fmk (20 μM) or BrdU (1.6 μM) was added to the basal medium.

Detection of apoptosis

Freshly dissected or after organotypic culture, the retinas were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.1. Apoptotic cell death was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), performed in whole-mount retinas, based on previous methods (Diaz *et al*. 1999) using FITC-dUTP and following the manufacturer's instructions (Promega). For more accurate scoring, the retinas were first dissociated with collagenase (55 U/mL), hyaluronidase (55 μg/mL), trypsin (1.6 mg/mL) and EDTA (2.7 mM) in a 20-min incubation at 37°C. The proportions of TUNEL-positive cells and pyknotic nuclei were scored after TUNEL staining and nuclear staining with 4′,6-diamidino-2-phenylindole, respectively.

Immunoblots

Akt, phospho-Akt and β-tubulin protein levels were determined by immunoblot. Cultured retinas were heated in Laemmli buffer (90°C for 10 min). The extracts were fractionated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using standard methods. Blots were incubated with the following primary antibodies: rabbit polyclonal anti-phospho-Akt (Ser473) 1 : 1000, goat polyclonal anti-Akt1 (1 : 1000) and β-tubulin (1 : 10 000). Blots were developed with the appropriate peroxidase-conjugated secondary antibody (1 : 20 000) using the Super Signal West Pico chemiluminescent substrate. Films were scanned and images were analysed using a GS-800 calibrate densitometer (Bio-Rad, Hemel Hempstead, Hertfordshire, UK).

Immunostaining of proliferating and differentiated cells

BrdU incorporation into DNA was used to determine proliferation. Briefly, embryonic retinas were cultured in the presence of 1.6 μM BrdU for 6 h. After culture, retinas were dissociated as above, and dissociated cells were incubated overnight at 4°C with anti-BrdU antibody (1 : 1000 dilution). βIII-Tubulin was used as a marker of differentiated neurons. Dissociated cells were incubated overnight at 4°C with the anti-βIII-tubulin antibody (1 : 5000 dilution; TUJ1).

Table 1 Primers used for PCR

Amplicon (bp)	Sequence (5′–3′)	T_m (°C)
Proinsulin (188 bp)	Fw: GGCTTCTTCTACACACCCA	58
	Rv: CAGTAGTTCTCCAGCTGGTA	
IGF-I (520 bp)	Fw: GTGGATGCTCTTCAGTTCGT	60
	Rv: ACACTCCTAAAGACGATGTT	
IR (850 bp)	Fw: TAGACCCGTGTTGCGGTTAAG	60
	Rv: GTGAGAGGAACGATCCAACG	
IGF-IR (977 bp)	Fw: TCTCTCTCTGGCCGACGAGT	58
	Rv: GAGCAGAAGTCACCGAATCG	
GAPDH (103 bp)	Fw: GCAATGCATCCTGCACCACC	58
	Rv: AGTGATGGCATGGACTGTGG	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-I, insulin-like growth factor I; IGF-IR, insulin-like growth factor I receptor; IR, insulin receptor.
GenBank accession numbers were NM008387 for mouse proinsulin 2, NM010512 for mouse IGF-I, JO5149 for mouse IR, AF056187 for mouse IGF-IR and NM001001303 for mouse GAPDH.

Staining was developed by incubation with the appropriate Texas Red-conjugated secondary antibody (1 : 200 dilution).

Immunostaining of cells for activated caspase 3

After culture, retinas were dissociated as above, and dissociated cells were incubated overnight at 4°C with anti-activated caspase 3 antibody (1 : 250 dilution). Staining was developed by incubation with an anti-rabbit Alexa 488-conjugated secondary antibody (1 : 200 dilution).

Scoring of apoptotic and differentiated cells and statistical analysis

For the quantification of apoptotic, proliferative, differentiated or activated caspase 3-positive cells, a minimum of 5000 cells were analysed per retina. ANOVA and Student–Newman *post hoc* tests were used for statistical analysis of the data. Median effective concentration (EC_{50}) values of the survival effect of the insulin family of growth factors were calculated by a non-linear regression of a four-parameter logistic model using ALLFIT software (De Lean *et al*. 1978). Statistical differences in EC_{50} values were calculated by the extra sum of squares principle (Cheng and Prusoff 1973).

RT-PCR determination

Total RNA was extracted from frozen retinas by homogenization in Trizol reagent according to the manufacturer's protocols. Genomic DNA was eliminated by deoxyribonuclease treatment. cDNA was synthesized by reverse transcription of 2.5 μg of total RNA using $oligo(dT)_{18-20}$ as a primer and the SuperScript first-strand synthesis system for RT-PCR. Negative controls were prepared for each RNA sample. The resulting cDNA was subjected to PCR with specific primers (Table 1). PCR mixtures contained 2 μL of cDNA, 2.5 U Taq DNA polymerase, 1 × PCR buffer, 3 μL of 25 mM $MgCl_2$, 1 μL of 10 μM deoxynucleotide triphosphates, 1 μL of 10 mM forward primer and 1 μL of 10 mM reverse primer in a total volume of 50 μL. After initial heating of the samples at 94°C for 2 min, the PCR amplification temperatures were 94°C for 30 s, 30 s at the respective annealing temperature and 72°C for 30 s [1 min for insulin receptor (IR) and IGF-IR]. In all cases, 35 cycles were performed, except for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (25 cycles). Final extension for all samples was at 72°C for 5 min. PCR products were electrophoresed in 1% (for IR and IGF-IR), 1.5% (for proinsulin and IGF-I) and 2% (for GAPDH) agarose gels and visualized by ethidium bromide staining. PCR products were verified by sequencing.

Insulin ELISA

An ultrasensitive mouse insulin ELISA was performed following the manufacturer's protocol. This ELISA recognizes insulin and proinsulin, although it is calibrated only for insulin. Pools of E15.5 retinas were deep frozen in dry ice, and then homogenized in 125 μL of the supplied 'calibrator 0' buffer by repeated passage through a syringe needle. To remove cell debris, lysates were centrifuged (20 000 *g*, 15 min, 4°C). Duplicates (50 μL) of the supernatants were used for assay.

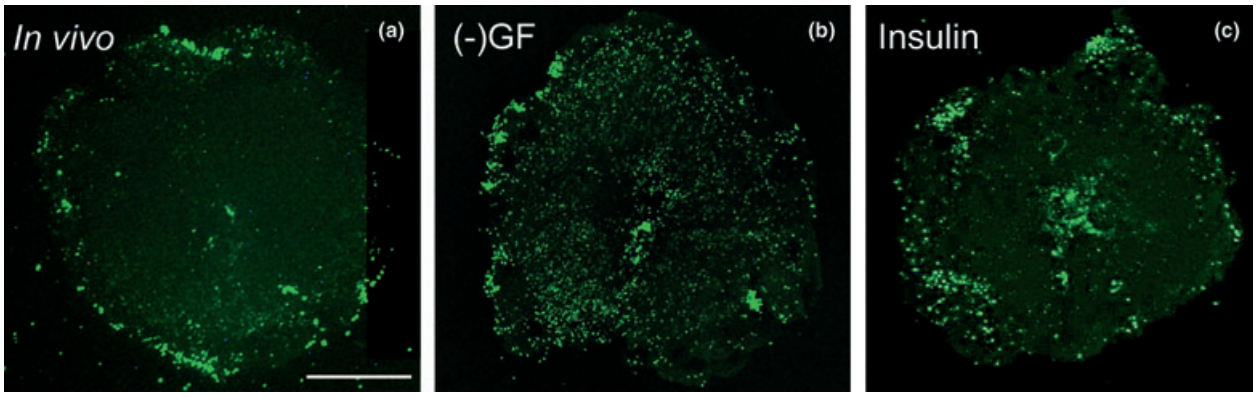

Fig. 1 Identification of apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining in embryonic day 15.5 (E15.5) mouse whole-mount retinas. Photographs are a compiled view from a whole-mount retina acquired by confocal microscopy. TUNEL-positive cells are visualized as small white spots. (a) Freshly dissected retina (*in vivo*). (b) Growth factor-deprived cultured retina [(–)GF]. (c) Insulin-treated cultured retina. Scale bar, 0.5 mm.

Results

Insulin-related growth factors support survival in the embryonic mouse retina

Programmed cell death, naturally occurring in the embryonic E15.5 mouse retina, was visualized by TUNEL staining in whole-mount retina (Fig. 1). Apoptotic cells were found to be concentrated around the optic nerve head and in the periphery (Fig. 1a). Scoring of TUNEL-positive cells in dissociated retinal cells yielded 1.4 ± 0.01% of total cells ($n = 9$), a number comparable with our previous observations in the chick embryo at similar stages.

Retinas cultured in chemically defined medium under growth factor deprivation conditions showed a four-fold increase in the number of apoptotic cells relative to that found *in vivo*, as quantified by TUNEL in whole-mount retina (Fig. 1b and Table 2). Growth factor deprivation-induced cell death in cultured organotypic retinas was reversed by 100 nM insulin, as the only growth factor added to the culture, to an extent and distribution similar to that observed *in vivo* (Fig. 1c and Table 2).

To determine the factor able to display this action in a physiological context, gene and protein expression analysis of proinsulin/insulin and IGF-I was performed throughout retinal development (Fig. 2). Proinsulin, IGF-I and their receptor mRNAs were found in the initial period of retinal neurogenesis, from E13.5 to E18.5 (Fig. 2a). We focused our attention on proinsulin/insulin, whose roles as neural growth factors have not been characterized in detail. The low proinsulin mRNA expression level yielded proinsulin/insulin immunoreactivity. E15.5 embryonic mouse retina contained 0.52 fmol of immunoreactive insulin per retina, as determined by specific ELISA. At this stage, the retina has a surface area of approximately 2 mm^2 (see whole mounts, Fig. 1) and an average thickness of 250 μm (data not shown); the extrapolated concentration is thus in the nanomolar range.

Analysis of the dose–response curves for proinsulin, insulin and IGF-I further supported their possible physiological role in the prevention of programmed cell death during retinal neurogenesis (Fig. 3a). Each single factor prevented cell death effectively, with comparable ED_{50} concentrations (0.3 ± 0.1 nM for proinsulin, 0.4 ± 0.3 nM for insulin and 0.8 ± 0.5 nM for IGF-I with pyknotic nuclei scoring; similar results were obtained for TUNEL-positive cell scoring). Thus, there is a good correlation between the ED_{50} determined and the previous extrapolated retinal concentration of proinsulin/insulin. Maximum survival was observed at 100 nM for all factors tested.

Table 2 Quantification of cell death in whole-mount embryonic day 15.5 (E15.5) mouse retina

Treatment	*n*	TUNEL-positive cells (relative values)
In vivo	11	23 ± 2
Growth factor-deprived	22	100 ± 4, ABC
Insulin (100 nM)	16	31 ± 3
Insulin (100 nM) + LY294002 (50 μM)	5	104 ± 9, ABC
Boc-D-fmk (38 μM)	3	25 ± 4

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining was performed on whole-mount retina (as shown in Fig. 1), and the total number of TUNEL-positive cells was scored directly by fluorescence microscopy with a × 20 objective. Letters indicate statistical differences, $p < 0.01$: A, versus freshly dissected (*in vivo*) retinas; B, versus Boc-D-fmk-treated retinas; C, versus insulin-treated retinas.

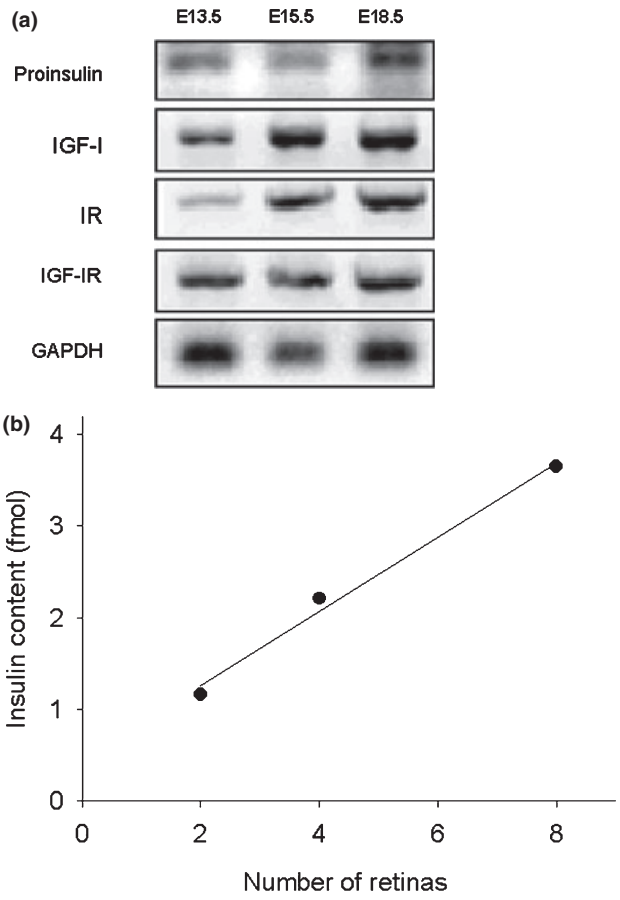

Fig. 2 Proinsulin/insulin, insulin-like growth factor I (IGF-I) and their receptors are expressed locally in embryonic mouse retinas. (a) RT-PCR analysis of proinsulin, IGF-I, insulin receptor (IR), IGF-I receptor (IGF-IR) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in embryonic mouse retinas of the indicated ages. Ethidium bromide-stained agarose gels are shown. (b) Retinal proinsulin/insulin content was quantified by ELISA. Pools of the indicated number of embryonic day 15.5 (E15.5) retinas were extracted in identical volumes and the proinsulin/insulin content was determined in duplicate. The regression line ($r = 0.993$) was employed to calculate the proinsulin/insulin content per retina.

The effects observed for each single insulin-related growth factor led us to test whether survival effects may be additive. Therefore, we compared single or combined treatments at 1 nM, a dose close to the respective ED_{50} concentrations (Fig. 3b). The survival effects of insulin and IGF-I were identical when added independently or together. In contrast, the proinsulin survival effect was further increased when insulin and IGF-I were also added to the culture medium.

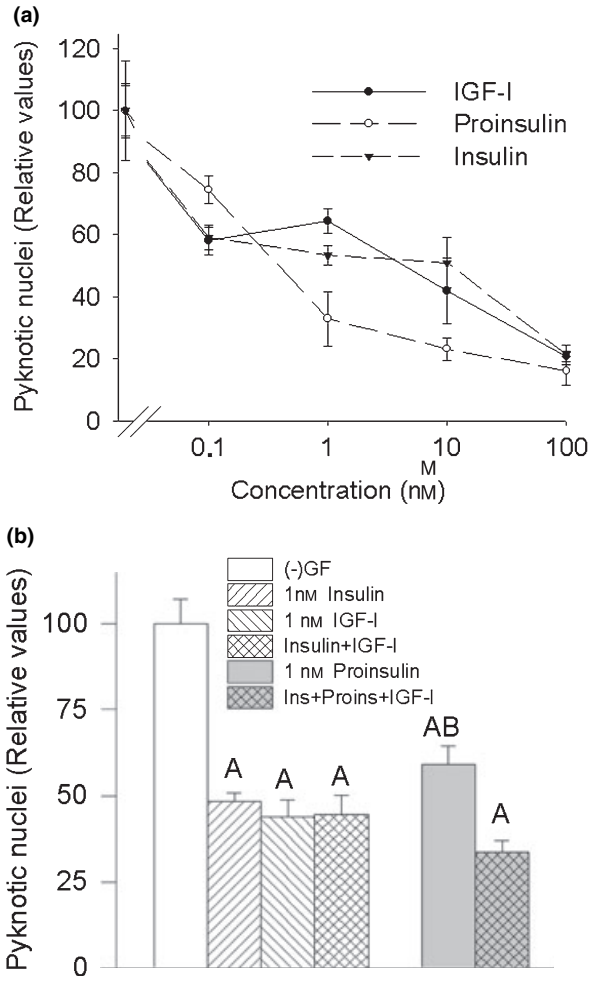

Fig. 3 Proinsulin, insulin and insulin-like growth factor I (IGF-I) attenuate cell death in embryonic day 15.5 (E15.5) mouse retinas. Embryonic retinas were cultured for 6 h in growth factor-free medium or with proinsulin, insulin or IGF-I at the indicated concentrations. After culture, retinas were dissociated and pyknotic nuclei were scored. (a) Dose–response curves. Median effective concentration (EC_{50}) values were calculated from the represented dose–response curves. (b) Combined treatments. Data, normalized with respect to the results obtained in retinas cultured in growth factor-deprived medium, represent the mean ± SEM (five retinas in a and eight retinas in b independently cultured for each experimental point). Apoptotic rate in growth factor-deprived conditions was 2.3% in (a) and 2.1% in (b). Letters indicate statistical differences, $p < 0.01$: A, versus retinas cultured in growth factor-deprived medium, B, versus retinas cultured in the presence of all growth factors together.

Phosphatidylinositol-3-kinase (PI3K) activation mediates the survival effects of insulin-related growth factors

The slightly different response to proinsulin relative to that to insulin and IGF-I prompted us to further characterize the intracellular survival pathways activated by the insulin-related growth factors. We employed 100 nM of each single factor to cover the whole range of the biological response. The survival effects of proinsulin, insulin and IGF-I at 100 nM were effectively blocked by the simultaneous presence of the PI3K inhibitor LY294002 (Fig. 4 and Table 2). Similar results were obtained when TUNEL-positive nuclei were scored. Therefore, PI3K activation appears to be the main biochemical pathway that mediates the survival effects of insulin family growth factors in the embryonic mouse retina.

Further, the phosphorylation of Akt, a serine/threonine kinase downstream of PI3K, was analysed using the same treatment. Retinas cultured for 6 h in the presence of insulin or IGF-I showed a six-fold increase in phospho-Akt level (Fig. 5), as well as a five-fold increase in the level of a 20-kDa phospho-protein detected by an antibody that recognizes proteins with a consensus sequence phosphorylated by Akt (data not shown). Concurring with the interference with

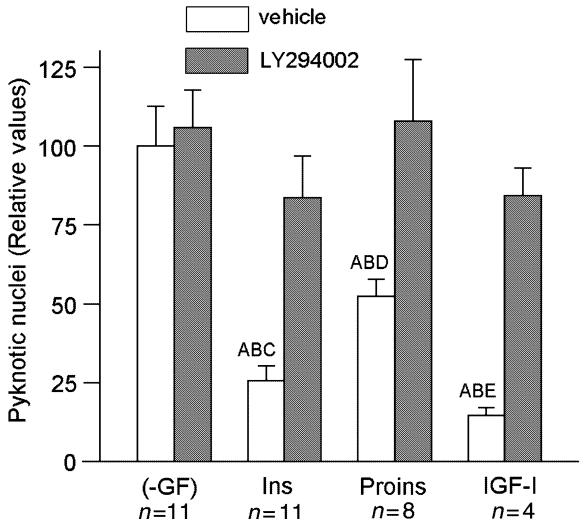

Fig. 4 Proinsulin (Proins), insulin (Ins) and insulin-like growth factor I (IGF-I) activate phosphatidylinositol-3-kinase (PI3K) in embryonic day 15.5 (E15.5) mouse retinas. Embryonic retinas were cultured for 6 h in growth factor-free medium or with 100 nM insulin, proinsulin or IGF-I alone or plus 50 μM LY294002. After culture, retinas were dissociated and pyknotic nuclei were scored. Letters indicate statistical differences, $p < 0.01$: A, versus retinas cultured in growth factor-deprived medium; B, versus LY294002-treated retinas; C, versus insulin plus LY294002-treated retinas; D, versus proinsulin plus LY294002-treated retinas; E, versus IGF-I plus LY294002-treated retinas.

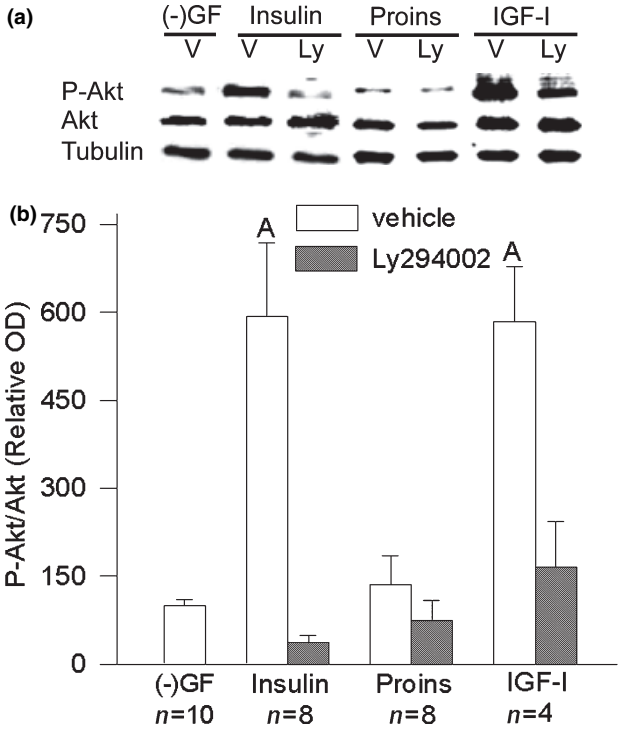

Fig. 5 Insulin and insulin-like growth factor I (IGF-I) trigger Akt phosphorylation in embryonic day 15.5 (E15.5) mouse retinas. Embryonic retinas were cultured for 6 h in growth factor-free medium or with 100 nM insulin, proinsulin (Proins) or IGF-I alone (V) or plus 50 μM LY294002 (Ly). After culture, extracts of individual retinas were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and immunoblotted with anti-phospho-Akt (p-Akt), anti-Akt and anti-tubulin antibodies. (a) Representative immunoblots. (b) Quantification of Akt phosphorylation relative to total Akt. Data are represented as the mean ± SEM of the densitometric values of the bands. Letters indicate statistical differences, $p < 0.01$: A, versus retinas cultured in growth factor-deprived medium. OD, optical density.

insulin and IGF-I survival effects (Fig. 4), LY294002 abolished insulin- and IGF-I-induced Akt phosphorylation (Fig. 5), as well as Akt substrate phosphorylation (data not shown), indicating that PI3K activation and downstream Akt phosphorylation are required for insulin and IGF-I action. Surprisingly, despite the fact that LY294002 also attenuated the proinsulin survival effect (Fig. 4), Akt phosphorylation was not increased in retinas cultured for 6 h in the presence of proinsulin (Fig. 5).

In order to confirm whether or not proinsulin-induced PI3K activation leads to Akt phosphorylation, a detailed time course of Akt phosphorylation was performed for each single insulin-related growth factor (Fig. 6). A rapid and sustained Akt phosphorylation was similarly induced by insulin and IGF-I (Figs 6a–d). In contrast, proinsulin was not able to induce Akt phosphorylation over the basal level at any of the studied time points (Figs 6e and f).

Caspases and cathepsins mediate cell death induced by growth factor deprivation

We further characterized the executor mechanisms involved in early neural cell death in the embryonic mouse retina and its regulation by insulin-related growth factors. Caspase involvement in induced cell death in cultured retinas was demonstrated using a broad-spectrum caspase inhibitor, Boc-D-fmk, which clearly decreased cell death (Fig. 7a and Table 2). The number of apoptotic cells was very close to that found *in vivo* by any of the quantitative methods employed (in all cases, TUNEL-positive cell scoring was also performed; data not shown). Selective caspase inhibitors were used to dissect the executor pathways. All markedly reduced cell death, including z-VEID-fmk, a selective inhibitor for caspase 6, z-DEVD-fmk, a selective inhibitor for caspase 3, and z-YVAD-fmk, a selective inhibitor for caspase 1, indicating multiple caspase involvement in the execution of cell death (Fig. 7a).

To analyse the possible hierarchies amongst these caspases, the presence of activated caspase 3 was determined and quantified (Figs 7b and c). It is noteworthy that all of the activated caspase 3-positive cells already showed pyknotic nuclei, whereas only half of the dying cells in growth factor deprivation conditions were positive for activated caspase 3 (Fig. 7c, and data not shown). As expected, the presence of activated caspase 3-positive cells was abolished in Boc-D-fmk-treated retinas, and greatly reduced in z-DEVD-fmk-treated retinas. The relative reduction of activated caspase 3-positive cells by z-VEID-fmk paralleled that of TUNEL-positive cells; in turn, there was no significant difference in the ratio of activated caspase 3-positive apoptotic cells relative to that in the untreated retinas, suggesting that caspase 6 acts independently of caspase 3 (Fig. 7c). z-YVAD-fmk provoked a modest reduction in activated caspase 3-positive apoptotic cells, suggesting that caspase 1 mainly acts independently of caspase 3 (Fig. 7c).

Cell death was not completely blocked by any of the inhibitors, indicating the existence of caspase-independent cell death, in addition to a complex network of executor caspases. To further demonstrate and analyse an alternative pathway, retinas were cultured with selective inhibitors of cathepsin B, z-FA-fmk, and cathepsin L, z-FF-fmk. Both inhibitors markedly decreased cell death (Fig. 8a). To analyse possible interactions between both executor pathways, the presence of activated caspase 3-positive cells was determined in z-FA-fmk- and z-FF-fmk-treated retinas (Figs 8b and c). The relative reduction in activated caspase 3-positive cells induced by cathepsin inhibitors paralleled that of TUNEL-positive cells. There were modest differences when the ratio of activated caspase 3-positive apoptotic cells relative to that in the untreated retinas was compared (Fig. 8c). Co-administration of z-DEVD-fmk together with z-FA-fmk or z-FF-fmk did not induce a further inhibition of

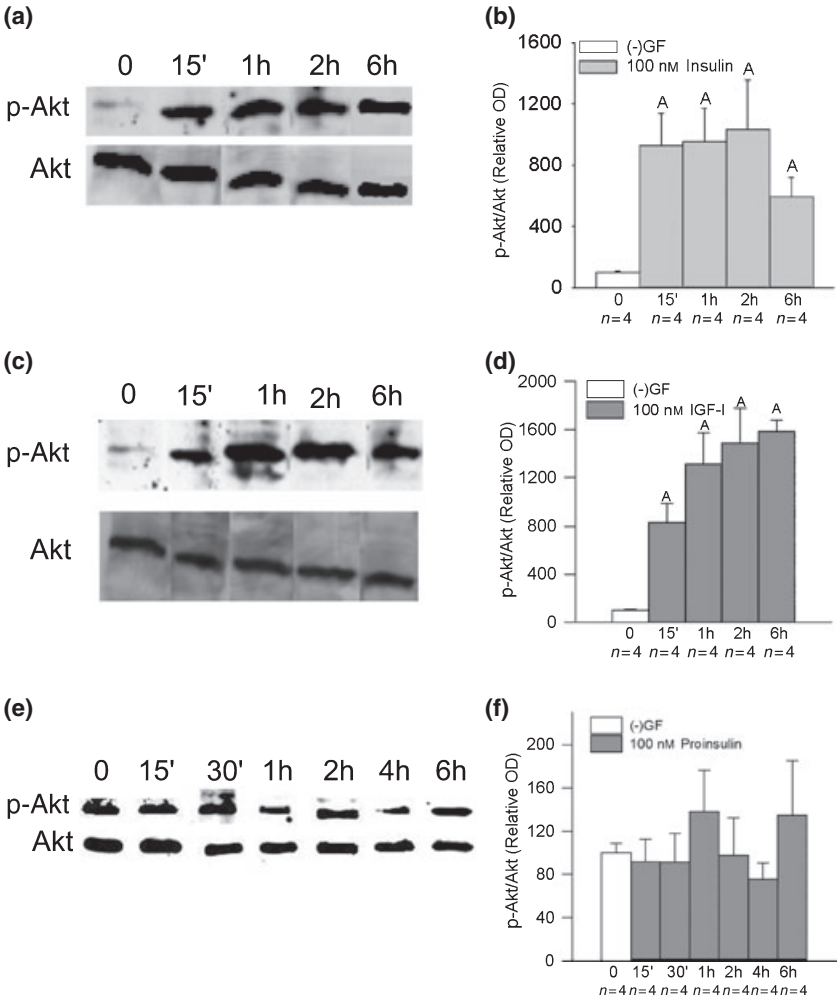

Fig. 6 Time course of Akt phosphorylation induced by proinsulin, insulin and insulin-like growth factor I (IGF-I) in embryonic day 15.5 (E15.5) mouse retinas. Embryonic retinas were cultured for the indicated period in growth factor-free medium or with 100 nM insulin, proinsulin or IGF-I. After culture, extracts of individual retinas were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and immunoblotted with anti-phospho-Akt (p-Akt) and anti-Akt antibodies. Representative immunoblots: (a) insulin; (c) IGF-I; (e) proinsulin. Quantification of Akt phosphorylation relative to total Akt: (b) insulin; (d) IGF-I; (f) proinsulin. Data are represented as the mean ± SEM of the densitometric values of the bands. Letters indicate statistical differences, $p < 0.01$: A, versus retinas cultured in growth factor-deprived medium. OD, optical density.

cell death compared with z-DEVD-fmk alone (data not shown). These observations suggest a contribution of cathepsins to cell death, although their action is mostly independent of the caspase 3 executor pathway.

Insulin prevents both caspase- and cathepsin-mediated cell death

To integrate our observations on the insulin survival effect and the executor pathways, retinas were cultured with insulin in combination with various inhibitors of these proteases (Fig. 9). Boc-D-fmk, z-FA-fmk and z-FF-fmk did not prevent cell death to a greater extent than insulin alone, indicating that insulin blocks the pathways leading to either caspase or cathepsin activation and/or execution.

Insulin prevents cell death affecting both proliferating neuroepithelial cells and young neurons

Neurons and glial cells are generated actively from proliferating neuroepithelial cells in E15.5 mouse retina. The populations affected by cell death were determined to further characterize the effects of insulin on the process of early neural cell death in the embryonic mouse retina. Of the apoptotic cells found in growth factor-deprived cultured retinas, 43.9 ± 4.5% ($n = 6$) also incorporated BrdU in the 6 h prior to death, and 61.1 ± 3.4% ($n = 6$) also stained for the neuronal marker βIII-tubulin. This indicates that both proliferating and differentiated populations are affected by cell death (Figs 10a–f). Insulin rescued proliferating cells and young neurons from death (Fig. 10g). In contrast, insulin had no significant effects in 6 h on the total number of BrdU- or βIII-tubulin-positive cells (54.8% and 7.8% of total cells, respectively, in growth factor-deprived conditions).

Discussion

In this study, we aimed to elucidate the role of the insulin family growth factors, including proinsulin, insulin and IGF-I, in the regulation of programmed cell death in a primary embryonic tissue, the mouse neuroretina. Our observations suggest the physiological relevance of proinsulin/insulin, as

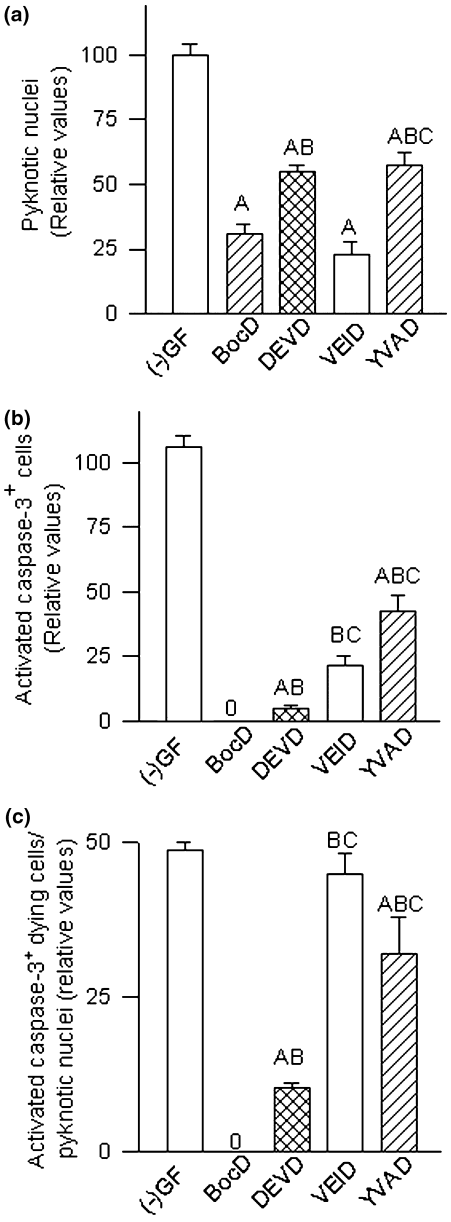

Fig. 7 Caspase inhibitors decrease programmed cell death in embryonic day 15.5 (E15.5) mouse retinas. Embryonic retinas were cultured for 6 h in growth factor-free medium or with caspase inhibitors (38 μM z-Boc-D-fmk, 150 μM z-DEVD-fmk, 38.7 μM z-VEID-fmk or 150 μM z-YVAD-fmk). After culture, retinas were dissociated and scored for pyknotic nuclei (a) or activated caspase 3-positive cells (b, c). Data are represented as the mean ± SEM ($n = 6$). The letters indicate statistical differences, $p < 0.01$: A, versus retinas cultured in growth factor-deprived medium; B, versus z-Boc-D-fmk-treated retinas; C, versus z-VEID-fmk-treated retinas. Apoptotic rate in growth factor-deprived conditions was 1.8%.

supported by their local production and potency, in the nanomolar range, in promoting survival. In addition, we characterized cell death distribution, the cell populations affected, and the signalling and executor pathways, all poorly understood features of early neural cell death.

Growth factor deprivation in organotypic retinal cultures induced cell death (Fig. 1b and Table 2), an observation that supports its trophic dependence during early stages of neural development (de la Rosa and de Pablo 2000). Proinsulin, insulin or IGF-I, added individually to the culture medium, effectively prevented cell death, reducing it to an extent similar to that observed *in vivo*. These observations concur with the mRNA and protein expression data (Fig. 2), and with our previous observations in the chick embryo (Diaz *et al*. 1999, 2000). Similarly, the expression of insulin family growth factors and their receptors has been described in rodents in the developing and adult central nervous system, including the retina (Thomopoulos and Pessac 1979; Das *et al*. 1984; Campana *et al*. 1999; Van Kleffens *et al*. 1999; Gleichmann *et al*. 2000; see also Varela-Nieto *et al*. 2003 for a review).

Insulin and IGF-I showed biphasic dose–response curves, whereas proinsulin showed a sigmoid curve (Fig. 3a). This finding may correlate with the availability of atypical, promiscuous, hybrid receptors able to bind proinsulin, insulin and IGF-I, as described in the embryonic chick retina (Garcia de Lacoba *et al*. 1999), together with canonical, selective, homodimeric insulin and IGF-I receptors. The small size of the mouse retina does not permit biochemical studies similar to those performed in the chick retina. Proinsulin probably acts by binding to atypical, hybrid receptors, whereas insulin and IGF-I bind to both canonical and atypical receptors. This differential behaviour was further supported by the fact that insulin and IGF-I did not show an additive response, whereas the proinsulin survival effect was further increased by simultaneous treatment with insulin and IGF-I (Fig. 3b). These results also suggest that insulin and IGF-I share a signalling pathway, whereas proinsulin appears to act through a slightly different survival pathway, starting at the receptor level.

The survival effect observed for insulin and IGF-I was mediated by PI3K and Akt activation (Figs 4–6). Activation of PI3K/Akt is a frequent step in the signalling pathway of the insulin-related growth factors (Varela-Nieto *et al*. 2003), although its involvement is not well defined in early neural cell death (Campana *et al*. 1999; Gleichmann *et al*. 2000). Through PI3K activation, IGF-I and insulin prevent serum deprivation-induced apoptosis in rat amacrine cells and in the R28 retinal cell line (Barber *et al*. 2001; Politi *et al*. 2001), and protect ganglion cells from death after optic nerve transection (Kermer *et al*. 2000b). The proinsulin survival effect also required PI3K, as it was prevented by LY294002, but, surprisingly, Akt did not appear as its downstream effector. Further work will be necessary to define the survival pathway downstream of PI3K in this system. The divergence in signalling may correlate with the observed differences in the survival effect of proinsulin with respect to insulin and IGF-I (Fig. 3), and may suggest a specific pathway for

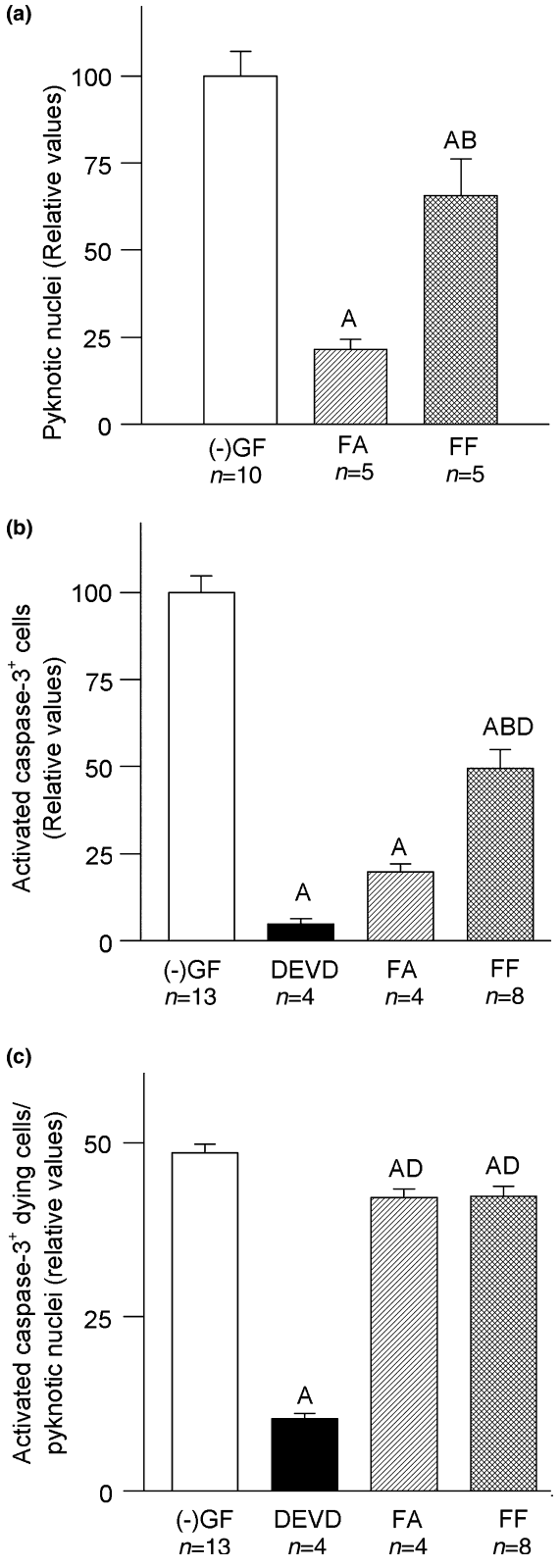

Fig. 8 Cathepsin inhibitors decrease programmed cell death in embryonic day 15.5 (E15.5) mouse retinas. Embryonic retinas were cultured for 6 h in growth factor-free medium or with cathepsin inhibitors (150 μM z-FA-fmk or 20 μM z-FF-fmk). After culture, retinas were dissociated and scored for pyknotic nuclei (a) or activated caspase 3-positive cells (b, c). Data are represented as the mean ± SEM. The letters indicate statistical differences, $p < 0.01$: A, versus retinas cultured in growth factor-deprived medium; B, versus z-FA-fmk-treated retinas; D, versus z-DEVD-fmk-treated retinas. Apoptotic rate in growth factor-deprived conditions was 1.6%.

hybrid receptors. To our knowledge, this is the first report studying the intracellular pathway activated by proinsulin in a physiological developing system.

Programmed cell death occurs naturally during early neurogenesis in the mouse retina (Laemle *et al*. 1999; Pequignot *et al*. 2003; this report). TUNEL analysis of whole-mount retina allowed improved determination of the dead cell distribution and better quantification than in previous studies (Fig. 1 and Table 2). The cell death distribution was unchanged following different treatments, despite numerical differences, with a central, intensely stained area, as well as peripheral locations. This suggests intrinsic susceptibility to death of certain cell populations, which is maintained in the relatively physiological conditions of the organotypic culture. Cell death execution was thus also investigated in this system. Caspase activation mediated cell death triggered by growth factor deprivation, as shown by the effect of caspase inhibitors in cultured organotypic retinas (Fig. 7). Caspases 6, 1 and 3 were involved in the execution of cell death. Although caspases 3 and 6 appeared to act independently, there was a minor level of interaction between caspases 1 and 3 in retinal cells. *In vitro* studies have shown that caspase 1 may activate caspase 3 (Van de Craen *et al*. 1999). Indeed, a complex network of caspases has been observed in adult retinas, as programmed cell death triggered by several insults is executed by more than one simultaneous cascade of caspases (Chaudhary *et al*. 1999; Katai and Yoshimura 1999; Hyun *et al*. 2000; Guimaraes *et al*. 2003). Caspase involvement in early retinal cell death concurs with the proposed role of caspases as executors of cell death in the nervous system. Knock-out mice for various caspases show strong phenotypes, with a large excess of neurons, brain malformations and perinatal lethality (Kuida *et al*. 1996, 1998; Honarpour *et al*. 2000; Kuan *et al*. 2000). Some of the defects can be detected during early neurogenesis, demonstrating the relevance of the early phase of cell death (discussed in de la Rosa and de Pablo 2000; Boya and de la Rosa 2005). Caspases 3, 9 and 2 are expressed in the developing vertebrate retina (Kojima *et al*. 1998; Donovan and Cotter 2002). To our knowledge, there is no description of caspase 6 and 1 expression in retinal cells during development. In adult mouse retinas, however, caspase 1 appears to be one of the main executors of cell death triggered by some pathological insults (Katai and Yoshimura 1999; Katai *et al*. 1999; Grimm *et al*. 2000; Oshitari and Adachi-Usami 2003; Zheng *et al*. 2004), whereas caspase 6

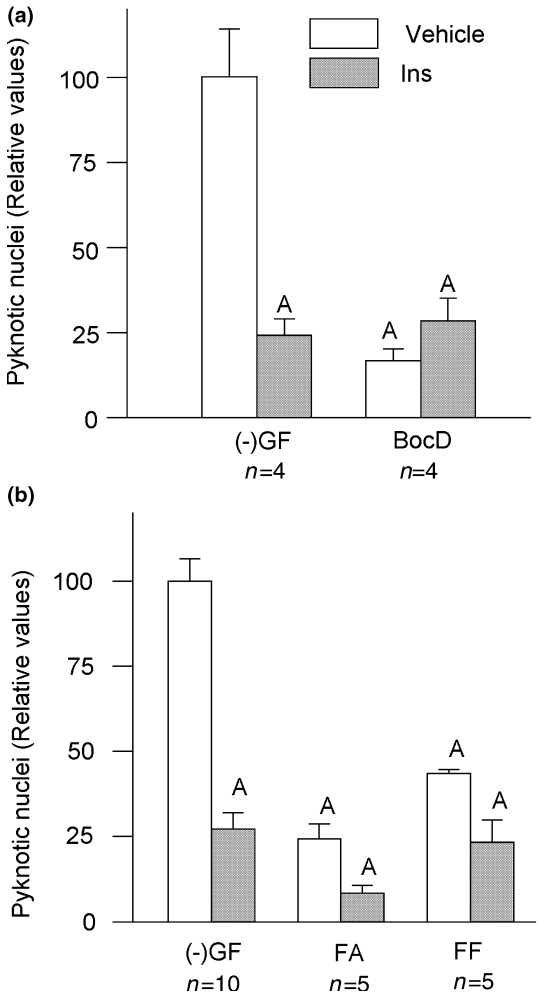

Fig. 9 Insulin prevents both caspase- and cathepsin-dependent programmed cell death in embryonic day 15.5 (E15.5) mouse retinas. Embryonic retinas were cultured for 6 h in growth factor-free medium or with insulin alone (100 nM) or in insulin combined with (a) caspase (38 μM z-Boc-D-fmk) or (b) cathepsin (150 μM z-FA-fmk or 20 μM z-FF-fmk) inhibitors. After culture, retinas were dissociated and scored for pyknotic nuclei. Data are represented as the mean ± SEM. Letter A indicates statistical differences, $p < 0.01$, versus retinas cultured in growth factor-deprived medium. Apoptotic rate in growth factor-deprived conditions was 2.1%.

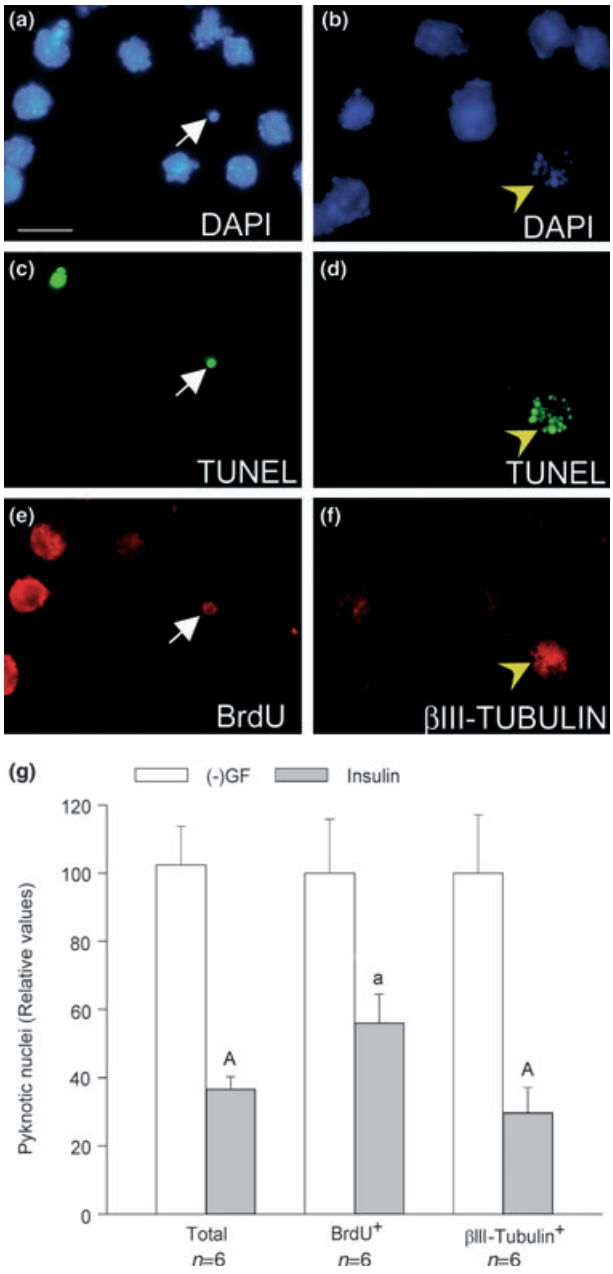

Fig. 10 Identification of apoptotic cells in embryonic day 15.5 (E15.5) mouse retinas. Embryonic retinas were cultured for 6 h in growth factor-free medium or with 100 nM insulin; 1.6 μM bromodeoxyuridine (BrdU) was also present in the culture medium. After culture, retinas were processed for terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining and BrdU and βIII-tubulin immunostaining in dissociated cells (a–f). Arrows show an apoptotic proliferating cell (a, c, e; same field). Arrowheads indicate an apoptotic neuron (b, d, f; same field). Scoring of double-labelled cells showed rescue of both proliferative and differentiated cells (g). Data are represented as the mean ± SEM. The letters indicate statistical differences versus retinas cultured in growth factor-deprived medium: a, $p < 0.05$; A, $p < 0.01$. Scale bar, 10 μm in (a)–(f). DAPI, 4′,6-diamidino-2-phenylindole.

appears to be involved in cell death following inhibition of protein synthesis in rat post-natal retinas (Guimaraes *et al.* 2003).

In addition to caspases, cathepsin B and cathepsin L were also involved in retinal cell death induced by growth factor deprivation (Fig. 8). Recent reports have also described the activation of caspase-independent pathways, including those of the cathepsins, calpains and granzyme A and B, in both physiological and pathological cell death processes (reviewed in Leist and Jaattela 2001; Jaattela and Tschopp 2003). Although less characterized than caspases in neural cell death, cathepsins are present in the nervous system; in

particular, cathepsin B and cathepsin L are expressed in the adult mammalian retina (Bernstein *et al*. 1989; Frohlich and Klessen 2001; Koike *et al*. 2003; Wasselius *et al*. 2003). Cathepsin B and L mRNAs are up-regulated in neuropathological conditions, such as Alzheimer's disease (Yoshiyama *et al*. 2000; Gan *et al*. 2004). Although the essential role of these two cathepsins for maturation and integrity of the postnatal central nervous system has been demonstrated by the phenotype of homozygous double mutant mice for cathepsin B and cathepsin L, which show brain atrophy (Felbor *et al*. 2002), their putative role as executors of developmental cell death has not been explored. This is therefore the first report demonstrating a clear involvement of these two cathepsins in the execution of developmental cell death in the nervous system.

Both caspase and cathepsin pathways are required for the efficient execution of cell death, as each selective inhibitor abolished cell death induced by growth factor deprivation (Figs 7 and 8). This may reflect the necessity of cross-activating interactions; however, these were not found at the level of caspase 3 (Fig. 8c). A role of cathepsins in caspase activation, either by direct cleavage or through Bid-mediated cytochrome *c* release, has been described in several models (Ishisaka *et al*. 1999; Stoka *et al*. 2001; Boland and Campbell 2004). The survival action of insulin affected both caspase- and cathepsin-dependent pathways (Fig. 9). Some previous direct and indirect data support caspase blockage by insulin family growth factors. IGF-I and insulin have been found to inhibit both caspase 1 activation and the subsequent cell death induced by growth factor deprivation in COS cells (Jung *et al*. 1996). In addition, neonatal transgenic mice overexpressing IGF-I show a reduction in cell death, caspase 3 expression and poly (ADP-ribose) polymerase (PARP) fragmentation in the cerebellum (Chrysis *et al*. 2001). IGF-I inhibits caspase 3 activation as well as apoptosis induced by several insults in primary cultures of dorsal root ganglion cells, and cortical and hippocampal neurons (Russell *et al*. 1998; Tamatani *et al*. 1998; Matsuzaki *et al*. 1999). In the retina, IGF-I protects axotomized rat retinal ganglion cells from secondary death via PI3K-dependent Akt phosphorylation and inhibition of caspases 3 and 9 (Kermer *et al*. 1998, 2000a), and insulin and IGF-I inhibit caspase 3 activation by a PI3K-dependent mechanism in the retinal cell line 28 (Barber *et al*. 2001). We nonetheless found no previous study in the nervous system showing the blockage of cathepsins, either B or L, by insulin family growth factors. Thus, this report unravels the interaction between these pathways.

Together, our data support a physiological involvement of insulin family growth factors, including proinsulin and insulin, in the regulation of programmed cell death, a genuine process of early neural development, and reveal a complex scenario in which specific regulatory pathways may act in specific cell populations during defined developmental processes.

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