IGF-I protects cortical neurons against ceramide-induced apoptosis via activation of the PI-3K/Akt and ERK pathways; is this protection independent of CREB and Bcl-2?

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Abstract

Current understanding of IGF-I-mediated neuroprotection implies the activation of phosphatidylinositol-3-kinase (PI-3K), which leads to the activation of Akt/Protein Kinase B. In non-neuronal cells, Akt phosphorylates and activates the transcription factor CREB, implicated in the transcription of the anti-apoptotic bcl-2 gene. This paper further analyses the anti-apoptotic IGF-I action in neurons. We show that IGF-I protects cortical neurons against ceramide-induced apoptosis. Ceramide decreases Akt phosphorylation during apoptotic process whereas a simultaneous treatment with IGF-I increases Akt phosphorylation. Analysis of the signal transduction pathways revealed that IGF-I induces CREB phosphorylation via PI-3K and ERK, whereas simultaneous ceramide and IGF-I treatment decreases CREB phosphorylation. Although an overexpression of Bcl-2 protects cortical neurons against ceramide-induced apoptosis, our data indicate that the Bcl-2 protein level is not modulated during IGF-I, ceramide and/or LY294002 treatment. In consequence, we demonstrated that IGF protects neurons against ceramide-induced apoptosis and that IGF-I protection involves the PI-3K/Akt and ERK pathways; this protection may be independent of CREB and Bcl-2.

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1. Introduction

Insulin-like Growth Factor-I (IGF-I) belongs to the somatomedin group of polypeptide hormones [12]. IGF-I has neuroprotective properties by reducing programmed death of motorneurons during development, after axotomy and spinal cord transection [34,35,42]. Intraventricular application of IGF-I has shown to attenuate neuronal cell loss after hypoxic–ischemic brain injury in adult rats [22,56,57]. In vitro studies revealed that IGF-I exerts its biological actions through a tyrosine kinase receptor, responsible for the phosphorylation of intracellular signal transduction proteins [59]. IGF-I-mediated neuroprotection is induced through the activation of the phosphatidylinositol-3-kinase (PI-3K), as demonstrated for cerebellar granule
cells and sensory dorsal root ganglion cells [1,3,39,50] leading to the phosphorylation and subsequent activation of Akt/Protein Kinase B [2,18,21,44]. Active Akt in turn can phosphorylate and thereby inactivate several cytoplasmic or nuclear targets such as the initiator caspase, caspase-9 [9,33], the proapoptotic protein Bad and the transcription factor FKHL1 [10,15,33]. A recent study in non-neuronal cells has demonstrated that Akt can also phosphorylate and activate the transcription factor CREB, implicated in the transcription of the antiapoptotic bcl-2 gene [49].

This report analyses the effects of the anti-apoptotic action of IGF-I on neuronal apoptosis using ceramide as an apoptotic inducer. Ceramide is a second messenger generated intracellularly by the sphingomyelinase-mediated cleavage of sphingomyelin [23,29,38]. Sphingomyelinase activity is strongly increased during developmental neuronal death in rat brain [54]. Increased ceramide levels are observed during apoptosis induced by growth factor-withdrawal in neurally differentiated PC12 cells [32] or cortical neurons [62] and after ischemia in the gerbil hippocampus [41]. Furthermore, apoptosis can be induced by exogenous ceramide treatment of PC12 cells [20,24], mesencephalic [7], hippocampal [40] or cortical neurons [60]. Recently, we have shown that ceramide regulates pro-apoptotic MAP kinase pathways and that JNK and p38 activation is crucial in ceramide-induced neuronal apoptosis in primary cortical neurons [60,61]. Thus, the objective of this study is to evaluate the protective effects of IGF-I on ceramide-induced neuronal cell death in primary cortical neuronal cultures.

2. Materials and methods

2.1. Materials

2.1.1. Drugs

C₂₋ceramide (N-acetylsphingosine, Biomol Research Lab, Plymouth Meeting, PA, USA) was prepared as a 25-mM stock in ethanol and incubated in the culture media. The specificity of C₂₋-ceramide was evaluated by comparing its effects with those of C₂₋-dihydro-ceramide (Biomol Research Lab). This analogue is lacking the 4–5 trans double bond in the sphingosine moiety of C₂₋-ceramide, and is unable to activate the sphingomyelin pathway in lymphocytes [4,45] and neurons [7].

Insulin-like growth factor-I (Sigma, Saint Louis, MO, USA) was prepared as 0.1 M acid acetic stocks at 0.05 mg/mL and added to the culture media as described. LY294002 (Cell Signaling Technology, Ozyme, France) was prepared as DMSO stocks at 50 mM and added to the culture media as described. ERK pathway inhibitor U0126 (Promega) was prepared as DMSO stocks at 10 mM and added to the culture media as described.

2.1.2. Antibodies

We used Cell Signaling Technology rabbit antisera specifically directed towards phospho-Ser⁴⁷³ Akt diluted 1:1000, phospho-Ser¹⁰⁸ CREB diluted 1:750 for Western blot or 1:500 for immunocytochemistry. We also used rabbit polyclonal antisera raised against synthetic peptides specific for Akt (phosphorylation-state independent; Cell Signaling Technology) diluted 1:1000, mouse monoclonal antisera raised against a recombinant Bel-2 (Santa Cruz, Santa Cruz, CA, USA) diluted 1:1000. Phospho-ERK (P-ERK) diluted 1:1000, ERK diluted 1:750 and CREB diluted 1:1000 antibodies were all rabbit antisera purchased from Cell Signaling Technology.

2.1.3. Mice

The Swiss mice were purchased from Janvier (Le Genest-St-Isle, France). Bel-2 transgenic mice were generated from our breeding colony, from mice kindly given by Dr. Martinou, University of Geneva, Switzerland. Expression of Bel-2 in these transgenic mice has been previously published [17,37]. Adequate measures were taken to minimize the number of animals used, pain or discomfort. All experiments conformed to French guidelines on the ethical use of animals and European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Genotyping

Genotyping for bcl-2 was performed by PCR using two primers to detect the human bcl-2 transgene: forward 5’-ATGAGCCTTGAGACTGAA-3’, reverse 5’-GAA-GACTCTGCTCAGTTTGGG3’. Cycling parameters were 5 min at 94 °C for one cycle, 45 s at 94 °C, 45 s at 60 °C, 1 min at 72 °C for a total of 40 cycles. PCR products were resolved on a 2% agarose gel.

2.3. Primary cultures of mouse cortex

Embryos were removed at day 14 from timed-pregnant Swiss mice (Janvier, Le Genest-St-Isle, France). The cortex was dissected, mechanically dissociated and plated on polyethyleneimine (1 mg/mL) coated culture wells, in Eagle’s basal medium (Eurobio, Les Ulis, France), supplemented with 5% horse serum (HS; Eurobio) and 2.5% fetal calf serum (FCS; Eurobio), at a density of 7 × 10⁵ cells/cm². After 2 days in culture the media was replaced with N5 medium [26] with 180 mg/L glucose and changed daily; FCS content was reduced to 1%; Ara-C (cytosine arabinoside, 3 μM, Sigma, Saint Louis, MO, USA) was added to prevent astrocyte proliferation (our cultures were at least 95% neuronal after this treatment), and MK-801 (1 μM, Research Biochemicals International, Natick, MA, USA) to prevent excitotoxicity [7,28].

Primary neuronal cultures taken from the bcl-2 transgenic mice were prepared using the cortex. The cortex from one embryo provides approximately 4 × 10⁶ cells that we
plated at a density of $7 \times 10^5$ cells/cm$^2$ in 96 well plates. Each culture was derived from an individual embryo, as genotyping was performed after putting the cells into culture.

### 2.4. Ceramide treatment

Embryonic cortical neurons cultivated in 5% HS and 1% FCS, optimal conditions for their maturation and differentiation were insensitive to c$_2$-ceramide treatment. That is why we reduced serum content to 1% HS, 24 h before any ceramide treatment [7]. Thus, the effects seen were not due to partial serum deprivation but rather to specific treatment, since control cells were in the same serum conditions during the same time as the treated cells. The culture conditions (concentration and time course) under which c$_2$-ceramide provokes cell death were determined by a cell-viability test.

### 2.5. Cell viability

Cell viability was determined by counting viable cells after a ten-min treatment with bisbenzimide (0.8 µg/mL), Triton X-100 (0.005%) or with the MTT assay (the conversion of the yellow tetrazolium salt [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT] to the purple formazan dye depends upon the mitochondrial activity [3,53]). The MTT assay was performed on neurons in N5 medium 1% HS, with MTT (0.5 mg/mL) for 4 h in a humidified atmosphere at 37 °C, 5% CO$_2$. The purple formazan salt was solubilized in 100 µL of a solution containing 0.1 M HCl in isopropanol. The spectrophotometric absorbency of the samples was determined at a 560-nm wave length.

### 2.6. Transfections

Transient transfection of primary cortical cultures was performed with LipofectAMINE 2000 (Invitrogen, Groningen, The Netherlands) as recommended by the manufacturer's protocol. Cells were transfected with 1 µg of a plasmid encoding enhanced green fluorescent protein (pEGFP-N3; Clontech, Cambridge, UK). After 6 h, the cultures were rinsed with fresh media. Transfection efficacy was 3–5% of the cells. After treatments, the cells were fixed as described for immunocytochemistry.

### 2.7. Western blot analysis

Cultured neurons were lysed in solubilization buffer (10 mM Tris–HCl, 50 mM NaCl, 1% Triton X-100, 30 mM Na$_2$PO$_4$, 50 mM NaF, 5 µM ZnCl$_2$, 100 µM Na$_3$VO$_4$, 1 mM dithiothreitol, 5 mM okadaic acid, 2.5 µg/mL aprotinin, 3.6 µM peptatin, 0.5 µM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 5.3 µM leupeptin) at 4 °C. Insoluble materials were removed by centrifugation (13,000 g for 20 min at 4 °C), supernatants were isolated and the samples were stored at −80 °C. Proteins were dosed with the DC protein assay kit (Bio-Rad, Hercules, CA, USA). Cell-extracts containing equivalent amounts of protein were boiled for 5 min in sample loading buffer. After a 10% (15% for Bcl-2 detection) SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (ICN Biochemicals, Costa Mesa, CA USA). The transfer was checked by Coomassie blue staining of the gels and the equal gel loading was confirmed by Ponceau red staining of the membranes. The transfer was also checked by the homogenous background of the blots after revelation. Non-specific sites were blocked with 5% skimmed dried milk for 2 h. Blots were then incubated overnight at 4 °C with primary antibodies against the phosphorylated form of the kinases, in 5% Bovine Serum Albumin (BSA). They were then incubated with horseradish peroxidase-conjugated secondary antibodies in 5% skimmed dried milk for 1 h. The revelation was processed with enhanced chemiluminescence substrate (Amersham, UK). The blots were stripped with 0.1 M glycine–HCl (pH 2.8) twice for 30 min at 60 °C and SDS 2% for 10 min, followed by saturation in 5% skimmed dried milk overnight at 4 °C. They were then incubated with the primary antibodies detecting both phosphorylated and non-phosphorylated forms of the kinases, in 5% skimmed dried milk, followed by incubation with the secondary antibody for 1 h, and revealed with the ECL kit as described above. Quantification was processed using the Densylab software (Microvision Instruments).

### 2.8. Immunocytochemistry

After c$_2$-ceramide treatment, cells were fixed with PBS (Phosphate Buffer Saline) containing 2% paraformaldehyde for 40 min, and then incubated with methanol/acetone solution (50/50) for 10 min at 4 °C. After washing three times with PBS, plates were treated with blocking buffer containing FCS 10%, BSA 3% Triton X-100 0.2% in PBS for 30 min. Polyclonal antibody raised against phosphorylated CREB was incubated overnight at 4 °C in PBS containing 1% BSA and 0.2% Triton X-100. Plates were rinsed 3 times in PBS and then incubated with an anti-rabbit Cy3-conjugated antibody (1:2000) for 1.5 h in PBS containing BSA 1%, Triton X-100 0.2%. After counterstaining with Hoechst, cells were mounted under coverslips using Vectashield (Vector Aneys, France).

### 3. Results

#### 3.1. IGF-I protects cortical neurons against ceramide-induced apoptosis

The effect of IGF-I on ceramide-induced apoptosis was examined in primary cortical neurons. Cortical neurons
were treated after a 5-day maturation period in vitro with c2-ceramide (40 μM) and IGF-I (10 ng/mL), then assayed for cell viability at various times after treatment using the MTT metabolism assay (Fig. 1A). By measuring survival, we have shown that IGF-I inhibited ceramide-induced apoptosis. From 16 h until 48 h of treatment, co-treatment of ceramide with IGF-I resulted in a survival increase from 61% to 97% at 24 h, and from 50% to 84% at 48 h (Fig. 1A). Furthermore, dose dependence analysis (Fig. 1B) reveals that a dose of 5 ng/mL completely protects cortical neurons from ceramide-induced apoptosis.

GFP transfection of the neurons allowed determination of their morphology during treatment. After 24 h of ceramide treatment, apoptotic features such as neurite fragmentation and nuclear condensation and fragmentation were observed (Figs. 1D and H). In IGF-I treated cells, GFP labeling visualized intact neurons with long neurites, as observed in the untreated control cells (Figs. 1C and E). When cells were co-treated with ceramide and IGF-I, cells looked healthy similar to control cells (Fig. 1F). In conclusion, we show that IGF-I protects neurons against ceramide-induced apoptosis, and that such protected neurons show no signs of nuclear fragmentation and condensation or neurite fragmentation.

3.2. Ceramide decreases P-Akt, whereas IGF-I increases it, even in presence of ceramide

In non-neuronal cells, ceramide has been shown to inhibit the PI-3K/Akt pathway. To determine whether this pathway is modulated during ceramide-induced neuronal apoptosis, Western blots of cellular extracts were performed with antibodies directed against the phosphorylated form of Akt. As ceramide inhibits Akt by promoting dephosphorylation of serine 473 [52] and phosphorylation of both residues is required for the optimal activation of Akt [31], we analyzed phosphorylation of serine 473. After 0.5 h, c2-ceramide treatment resulted in a rapid and substantial decrease in the signals corresponding to P-Ser473-Akt (Fig. 2A, first panel) without significantly affecting the level of total Akt (Fig. 2A, second panel). The ratio P-Akt/total Akt decreased very rapidly during c2-ceramide treatment and reached 15% of the control value after a 0.5-h treatment, this low phosphorylation level remained throughout the apoptotic process (up to a 24-h treatment; Fig. 2B).

In contrast, a 0.5-h co-treatment of c2-ceramide and IGF-I caused a rapid and substantial increase in signals corresponding to P-Ser473-Akt (Fig. 2A, third panel) without significantly affecting the level of total Akt (Fig. 2A, fourth panel).
The ratio P-Akt/total Akt increased very rapidly during c2-ceramide and IGF-I co-treatment and reached 304% of the control values after a 0.5-h treatment. This increase in phosphorylation level was maintained throughout the treatment (up to 24 h; Fig. 2B). Inhibition of PI-3K by LY294002 abolishes IGF-I protection of cortical neurons against ceramide-induced apoptosis.

To determine whether the PI-3K/Akt pathway is involved in IGF-I protection against c2-ceramide-induced neuronal cell death, we applied the PI-3K inhibitor LY294002. This inhibitor is specific for PI-3K [13]. In Fig. 2C, ceramide decreased survival to 57% of the control and IGF-I protected neurons against ceramide-induced neuronal apoptosis, as detailed in Fig. 1. LY294002 (50 μM) alone decreased survival to 40% of the control. LY294002 did not enhance ceramide-induced apoptosis after 24 h of treatment, but abolished IGF-I protection of cortical neurons against ceramide-induced neuronal apoptosis. Western blots of cellular extracts with the same treatments were performed with antibodies directed against the Ser473 phosphorylated form of Akt (Fig. 2D). We observe that when survival level was similar to the control value (control, IGF-I, cer+IGF-I), Akt was phosphorylated, whereas when apoptosis was induced (cer, LY, IGF-I+LY, cer+LY, cer+IGF-I+LY), the level of Akt phosphorylation was low or indetectable.

3.3. Ceramide causes a decrease in CREB phosphorylation in cortical neurons

As Akt may phosphorylate and activate CREB, we tested whether the phosphorylated form of CREB (P-CREB) was modulated during ceramide and/or IGF-I treatment. Western blot analyses of cellular extracts were performed after 7 h of treatment with ceramide and/or IGF-I (Fig. 3A), the time corresponding to the highest level of Akt phosphorylation during IGF-I and ceramide treatment, and also the minimum level of Akt phosphorylation during ceramide treatment alone (Fig. 2B). Ceramide treatment decreased CREB phosphorylation compared to control values, whereas IGF-I treatment increased CREB phosphorylation. Thus IGF-I is able to induce CREB activity. However, when ceramide and IGF-I were applied in tandem, CREB phosphorylation decreased to the same level as the one after ceramide treatment alone. We also performed a more detailed kinetic analysis of P-CREB during ceramide and ceramide+IGF-I treatments, but there was no difference in the level of CREB phosphorylation at any time between both treatments (data not shown). Furthermore, we performed a P-CREB analysis after 1 h of the same different treatments as presented in Fig. 3A, and we got the exact same results as the one shown in Fig. 3A (data not shown).

Immunocytochemical detection of P-CREB confirmed these results (Figs. 3B–I). A basal level of P-CREB immunoreactivity was found in the nuclei of control cells (Figs. 3B and J), whereas after 7 h of c2-ceramide (40 μM) treatment, P-CREB immunoreactivity was distinctly decreased (Figs. 3C and J). After IGF-I treatment, P-CREB immunoreactivity was increased (Figs. 3D and J), whereas ceramide+IGF-I treatment (Figs. 3E and J) decreased.
immunoreactivity to the level of ceramide treated cells. Thus, CREB phosphorylation is not involved in the IGF-I protection against ceramide-induced neuronal apoptosis.

### 3.4. The PI-3K/Akt pathway is involved in IGF-I-induced phosphorylation of CREB

To test if the PI-3K/Akt pathway is involved in CREB phosphorylation, we performed Western blot analyses of cellular extracts treated with ceramide, IGF-I and/or LY294002 during 7 h (Fig. 3A). These experiments revealed that LY294002 treatment alone, or in tandem with ceramide, had no effect on CREB phosphorylation. We also showed that LY294002 inhibited IGF-I induced-phosphorylation of CREB (compare IGF to IGF+LY treatments in Fig. 3A), but did not further decrease CREB phosphorylation after cer+IGF+LY treatment, compared to cer+IGF-I treatment. In conclusion, PI-3K is involved in IGF-induced phosphorylation of CREB but PI-3K inhibition is not involved in the basal phosphorylation of CREB, nor in CREB phosphorylation decrease induced by ceramide.

### 3.5. The ERK pathway is involved in IGF-I-induced phosphorylation of CREB and in IGF-I-mediated protection of cortical neurons

To investigate how ceramide antagonizes the stimulatory actions of IGF-I on CREB, we performed Western blot analyses of cellular extracts treated with IGF-I, c2-ceramide and/or the ERK pathway inhibitor, U0126, during 7 h. We showed that IGF-I increases CREB phosphorylation (Figs. 3A, J and K), and that U0126 treatment decreases CREB phosphorylation, even in the presence of IGF-I (Fig. 3K). We also showed that IGF-I-induced ERK phosphorylation is abolished by U0126. In addition, we showed that ceramide or U0126, with or without IGF-I, induces a decrease in P-CREB phosphorylation (Figs. 3A and K). In conclusion, the ERK pathway is involved in the basal and in IGF-I-induced phosphorylation of CREB.

To see if the ERK pathway is also involved in the survival of cortical neurons, we performed survival experiments after treatment with IGF-I, c2-ceramide and/or the ERK pathway inhibitor, U0126, during 24 h. As we have already shown [60], inhibition of the ERK pathway does not affect neuronal survival in control conditions, nor does it affect ceramide-induced apoptosis (Fig. 3L). We showed here that U0126 abolishes IGF-I-mediated protection against ceramide-induced apoptosis (compare cer+IGF-I and cer+IGF-I+U0126 in Fig. 3L). Thus, the ERK pathway is involved in IGF-I-mediated protection of cortical neurons against ceramide-induced apoptosis.

### 3.6. Bcl-2 is not involved in cortical neurons protection against ceramide-induced apoptosis

As the transcription factor CREB may regulate bcl-2 gene, we examined the effect of Bcl-2 on ceramide-induced apoptosis in primary cortical neurons from transgenic mice.
expressing human bcl-2 and their wild-type littermate. After a 5-day maturation period in vitro, cultures were treated with c2-ceramide (40 μM) and assayed for cell viability 24 h after treatment by counting cells with normal nuclei labeled with bisbenzimide (Fig. 4A). Our results show that bcl-2 overexpression inhibited ceramide-induced apoptosis and protected cortical neurons: 91% bcl-2 overexpressing neurons survived compared to 59% of the wild type. Thus, Bcl-2 protects cortical neurons against ceramide-induced apoptosis. The same cellular extracts as in Fig. 3A were submitted to Bcl-2 detection by Western blot (Fig. 4B). We showed that Bcl-2 protein level was not modulated by ceramide, IGF-I, or LY294002. In all conditions tested, the Bcl-2 protein level was comparable to the one of the control. We also performed a more detailed kinetic analysis of Bcl-2 during ceramide and ceramide+IGF treatments. As a result, we did not see any difference in the level of Bcl-2 protein at longer time points between both treatments (data not shown). Furthermore, we performed Bcl-2 analysis after 1 h of the same different treatments as presented in Fig. 4B, and we got the exact same results as the one shown in Fig. 4B (data not shown). Thus, although overexpression of Bcl-2 protects cortical neurons against ceramide-induced apoptosis, there is no modulation of Bcl-2 protein level in IGF protection against ceramide-induced apoptosis.

4. Discussion

The objective of this study was to investigate the effects of IGF-I on ceramide-induced neuronal apoptosis, and the possible link between this molecule and the PI-3K/Akt/CREB/Bcl-2 pathway in cortical neurons.

We showed for the first time that IGF-I protects cortical neurons from ceramide-induced apoptosis. IGF-I protection from ceramide has previously been shown in non-neuronal cells [30,36]. IGF-I has been also shown to protect hippocampal neurons from β-amyloid peptide toxicity [16], dorsal root ganglion neurons from Nerve Growth Factor withdrawal [59] and cortical neurons from hypoxia [58].

Pugazhenthi and his coworkers have proposed a link between IGF-I and the PI-3K/Akt/CREB/Bcl-2 pathway in cortical neurons [48,49]. To test this hypothesis in cortical neurons, we analyzed Akt phosphorylation during ceramide treatment with or without IGF-I and PI-3K inhibitor. Using these different treatments, we have shown that when survival level was similar to the control value, Akt was phosphorylated, whereas when survival was low, Akt phosphorylation level was also low or undetectable. Thus, Akt seems to be a key enzyme for cortical neurons survival. A minimal activation of the PI-3K pathway is required for the survival of neurons; indeed, the levels of neuronal survival are in agreement with the immunoblotting raised against phospho-Akt (taken as a test of PI-3K activity) in the control situation and after treatment by LY alone: when PI-3K pathway is inhibited, the neuronal survival is decreased. The inhibition of Akt by ceramide may be mediated by several processes. Ceramide may decrease Akt phosphorylation by inhibiting PI-3K or PDK2, kinases upstream of Akt. This might be the case in our model, since (i) Akt has a low undetectable level of phosphorylation during ceramide treatment with or without LY294002; and (ii) IGF-I-induced phosphorylation of Akt has been shown to involve PI-3K and PDK2 [14,25,43,46]. However the intermediates between ceramide and PI-3K or PDK2 have not been identified yet. We cannot exclude other mechanisms for ceramide-decreasing Akt phosphorylation, such as dephosphorylation of Akt by a Protein Phosphatase-2A-like phosphatase (PP2A), Ceramide Activated Protein Phosphatase (CAPP) [51] or PKCζ [5].

Since IGF-I and Akt promote neuronal survival, we examined a possible downstream effector, the transcription factor CREB. In accordance with previous results on non-neuronal cells [47,49], we have shown for the first time in neurons, that IGF-I induced CREB phosphorylation via PI-3K and ERK. However, our data demonstrate that CREB and Akt phosphorylations are not coupled, and that the neuronal survival is independent of CREB. Indeed, during ceramide and IGF-I treatment, neurons are protected, Akt phosphorylation increased but CREB phosphorylation decreased. Moreover, our experiments revealed that ERK is involved in the basal and in the IGF-I-induced phosphorylation of CREB. We also showed that IGF-I-mediated protection against ceramide-induced apoptosis involves the ERK pathway but not CREB. Thus, CREB is not linked to neuronal survival.

Our data showed that cortical neurons overexpressing Bcl-2 were protected from ceramide-induced apoptosis. A protective role of Bcl-2 from ceramide-induced apoptosis has previously been observed in two neuronal cell lines [19,63] probably by blocking the release of cytochrome c from the mitochondria into the cytosol [27,62]. However, immunoblotting experiments of Bcl-2 showed that Bcl-2 expression was not modulated during IGF-I, ceramide or LY294002 treatments. In consequence, Bcl-2 is not involved in IGF-I mediated protection. From this experiment we can conclude, that Akt does not act via CREB or Bcl-2 to protect neurons against ceramide-induced apoptosis. Akt may act on other proteins such as the transcription factor Forkhead or cytoplasmic proteins Bad, caspase-9 or Iκ-B Kinase (for review see [6,8,11]). Moreover, a recent study shows that ceramide may act on Bad and Forkhead during neuronal apoptosis [55].

To summarize our results, in control conditions, the PI-3K/Akt pathway is involved in the survival of cortical neurons and the ERK pathway is involved in CREB phosphorylation. When neurons are treated with IGF-I, the ERK and PI-3K/Akt pathways are involved in CREB phosphorylation. When ceramide induces neuronal apoptosis, the ERK and PI-3K/Akt pathways, as well as CREB, are inhibited. We demonstrate for the first time that IGF-I protects neurons against ceramide-induced neuronal apopto-
sis and that this protection involves the ERK and PI-3K/Akt pathways, but may be independent of CREB and Bcl-2.

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