Growth Differentiation Factor-15 Prevents Low Potassium-induced Cell Death of Cerebellar Granule Neurons by Differential Regulation of Akt and ERK Pathways*

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Growth differentiation factor-15 (GDF-15) is a novel member of the transforming growth factor-β superfamily and has been shown to be induced in neurons subsequent to lesions. We have therefore begun to study its putative role in the regulation of neuron survival and apoptosis. Cultured cerebellar granule neurons (CGN) survive when maintained in high K⁺ (25 mM) but undergo apoptosis when switched to low K⁺ (5 mM). GDF-15 prevented death of CGN in low K⁺. This effect could be blocked by phosphatidylinositol 3-kinase/Akt pathway inhibitors LY294002 or wortmannin. In contrast, mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK) pathway inhibitors U0126 and PD98059 potentiated GDF-15 mediated survival and prevented cell death in low K⁺ even without factor treatment. Immunoblots revealed GDF-15-induced phosphorylation of Akt and glycogen synthase kinase-3β. This activation was suppressed by phosphatidylinositol 3-kinase inhibitors. Low K⁺ induced delayed and persistent ERK activation, which was blocked by MEK inhibitors or GDF-15. ERK activation induced c-Jun, a member of the AP-1 transcription factor family. GDF-15 or U0126 prevented c-Jun activation. Furthermore, we show that GDF-15 prevented generation of reactive oxygen species, a known activator of ERK. Together, our data suggest that GDF-15 prevents apoptosis in CGN by activating Akt and inhibiting endogenously active ERK.

Transforming growth factor-βs (TGF-βs)1 comprise a superfamily of contextually acting cytokines with a broad array of biological activities. Depending on the developmental stage and cell type TGF-βs regulate diverse processes including development, cell proliferation, differentiation, survival, and death (1–4). Members of the TGF-β superfamily can be subdivided into subfamilies of structurally closer related proteins, including the TGF-βs 1–3, bone morphogenetic proteins, the growth/differentiation factors (GDFs), activins, and the glial cell line-derived neurotrophic factor (GDNF) family (5–7).

All TGF-β family members, except members of the GDNF subfamily, signal through heteromeric complexes of type I and type II serine/threonine kinases receptors. Within the type I and type II receptor families there are several subgroups, e.g. bone morphogenetic protein receptors, depending on their respective ligands (for reviews, see Refs. 7 and 8). GDNF, neurturin, persephin, and artemin signal through a heteromeric receptor complex consisting of the receptor tyrosine kinase Ret (9, 10) and glycosylphosphatidylinositol-linked α-receptors (11).

We and others (12, 13) have recently cloned a novel member of the TGF-β superfamily, GDF-15/macrophage inhibitory cytokine-1 (MIC-1). The protein does not belong to one of the known TGF-β subfamilies and represents a divergent member of the TGF-β superfamily. GDF-15 is widely synthesized in the central nervous system, most strongly expressed in the choroid plexus, and secreted into the cerebrospinal fluid, from where it may penetrate into the brain parenchyma (14–16). We have previously shown survival promoting effects of GDF-15 on unlesioned and intoxicated midbrain dopaminergic neurons in vitro and in vivo (15). Serotonergic neurons of the embryonic rat raphe were one additional neuron population that responded to GDF-15 by an increase in transmitter synthesis and uptake. However, motoneurons of the spinal cord and sensory dorsal root ganglionic neurons did not respond at all or only very moderately to GDF-15. At this point, it is not clear whether other types of central nervous system neurons are affected by GDF-15. In a previous study, we had also shown that GDF-15 protein is up-regulated in cortical neurons subsequent to a cold lesion (16). This may indicate functions of the protein in neurons affected by injury and suggest a role of GDF-15 in the execution of either survival or cell death programs.

Cultured cerebellar granule neurons (CGN) from postnatal rat represent a highly homogeneous neuron population that survive for weeks in vitro and develop characteristics of mature CGN in vivo when maintained in depolarizing concentrations of K⁺ (25 mM), but undergo apoptosis when cultured in physiological low K⁺ (5 mM) conditions (17–19). Although mechanisms underlying CGN apoptosis are not clear as yet, a requirement of RNA/protein synthesis, generation of reactive oxygen species (ROS), activation of caspases, and phosphorylation of c-Jun have been implicated in this apoptotic model (18, 20, 21).
Our previous observation that GDF-15 is specifically up-regulated in lesioned neurons prompted us to investigate pro-
or anti-apoptotic effects of GDF-15 and underlying mechanisms in CGN. Here we demonstrate that (i) GDF-15 prevents death of CGN in low K⁺ by activating Akt and down-regulating ERK, (ii) low K⁺-induced ERK activation, in turn, induces c-Jun, which can be inhibited by GDF-15, and (iii) GDF-15 mediated survival is accompanied by prevention of low K⁺-induced ROS generation in CGN.

EXPERIMENTAL PROCEDURES

Materials—Insulin-like growth factor-1 (IGF-1) was purchased from Ciba Geigy (Basel, Switzerland). The Eagle’s basal medium with Earle’s salts, glutamine, penicillin, streptomycin, trypsin, phosphate-buffered saline, and Hanks’ balanced salt solution were from Invitrogen (Karlsruhe, Germany). Culture plates were from Falcon. Fetal calf serum was obtained from Seromed (Berlin, Germany). LY294002, wortmannin, rabbit polyclonal antibodies (anti-phospho-Akt (Thr-308), anti-Akt, anti-phospho-p44/42 (ERK1/2), mitogen-activated protein kinase (Thr-202/Tyr-204), anti-ERK1/2, anti-phospho-e-c-Jun (Ser-63), anti-phospho-JNK (Thr-183/Tyr-185)), and the anti-rabbit IgG-HRP conjugated antibody were from New England Biolabs GmbH (Frankfurt, Germany). p44/42 MAP Kinase (Thr-202/Tyr-204), anti-phospho- Akt, anti-phospho-p44/42 (ERK1/2), and the anti-rabbit IgG-HRP conjugated antibody was a gift from Prof. Kersten Krieglstein (Göttingen, Germany). The U0126, PD98059, dead end colorimetric apoptotic assay kit, and Cyto Tox 96 assay for lactate dehydrogenase (LDH) measurements were from Promega (Madison, WI). Polyvinylidene difluoride membrane and the ECL chemiluminescence kit were from Amersham Biosciences Europe GmbH (Freiburg, Germany). The 2', 7'-dichlorodihydrofluorescein (DCF-H2) was from Molecular Probes. All other chemicals were purchased from Sigma.

Expression of Recombinant Human GDF-15—Full-length GDF-15 cDNA was cloned and sequenced as described previously (13). Recombinant human GDF-15 protein was expressed in baculovirus-infected insect SF9 cells and purified from supernatants as described previously (15). Protein extracts of uninfected cells were treated under the same conditions and used in parallel for controls.

Cell Cultures—Cerebellar granule neurons were isolated and cultured from 8 day old Wistar rats as described previously (22) with slight modifications. Briefly, the freshly dissected cerebellum was trypsinized and triturated in ice cold Ca²⁺- and Mg²⁺-free Hank’s balanced salt solution. The cells were resuspended in high K⁺ medium (Eagle’s basal medium containing 10% fetal calf serum, 25 mM KCl (Sigma), 2 mM glutamine, and 0.5% (v/v) penicillin/streptomycin). Cells were seeded in poly-l-lysine (100 µg/ml, Sigma) precoated wells in 12-well plates at an average density of 1,500 cells/mm². Cultures were incubated at 37 °C with 5% CO₂ in a humidified chamber. Cytosine arabinoside (10 µM, Sigma) was added after 24 h in vitro to prevent proliferation of non-neuronal cells. On day 4 in vitro the culture dishes were switched from high K⁺- to low K⁺-medium (Eagle’s basal medium, 5 mM KCl, 2 mM glutamine, and 0.5% penicillin/streptomycin; “5K”). Depending on the experiment at the time of medium change the cells in 5K were treated in the presence or absence of stimulants for indicated time points and were processed for the assays described below. All the cell death analyses were performed after 24 h of treatment.

LDH Measurements—LDH activity was assayed using an LDH assay kit (Promega). Total LDH release (percent) was calculated from maximum release, defined as the amount of LDH obtained after exposure of 25 mM K⁺ cultures to Triton X-100, 0.1% for 10 min at 37 °C. Propidium Iodide Staining—Propidium iodide (PI) was used to determine numbers of dead cells. PI (4.6 µg/ml) was added directly to the culture medium for 3 min at room temperature, and the cells were counted after fixation with 4% paraformaldehyde. Typically around 70% of the cells were counted in four randomly selected fields per dish. Numbers of PI-stained cells in low K⁺ medium were normalized to 100%. Cell counts were presented as percentages of low K⁺-induced cell death.

TUNEL (Terminal Deoxyuridinyltransferase Nick End Labeling) Assay—The TUNEL assay was performed according to the manufacturer’s manual (Promega). Around 500 cells in four different fields consisting of ~20–30% of the total culture were counted per coverslip under phase contrast microscope. Cell numbers were converted into percentage of apoptotic cells calculated from total cell numbers.

Reactive Oxygen Species (ROS) Measurement—ROS measurements were essentially carried out as described in Ref. 20. The culture plates were read on Fluostar OPTIMA plate reader (BMG Laubbach, Offenburg, Germany) at 480 nm excitation and 520 nm emission. Neurons grown in high K⁺ showed negligible fluorescence and were used for background fluorescence.

Western Blot Analysis—Neurons cultured in 12-well plates were harvested in 1 × SDS lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% w/v bromophenol blue. Cell lysates containing equal amounts of protein (25 µg/lane) were loaded on 10 or 12% SDS-polyacrylamide gels. The separated proteins were transferred onto a polyvinylidene difluoride membrane using a wet transfer system (Amersham Biosciences). The membranes were blocked with blocking buffer containing 5% dry milk in Tris-buffered saline with Tween 20 (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20) and incubated overnight with different primary antibodies. Incubations with secondary antibodies were performed for 1 h, and the antibody bands were detected with the enhanced chemiluminescence (ECL, Amersham Biosciences) reagents in accordance to the manufacturer’s protocol. Densitometric quantification of Western blots was done using NIH image (version 1.61) software.

RESULTS

GDF-15 Prevents Cell Death of CGN Induced by Low K⁺—We first assessed the effect of GDF-15 on CGN viability by monitoring the release of LDH into the culture medium. Fig. 1A (left panel) shows that GDF-15 significantly decreased release of LDH from CGN induced by low K⁺ (5K) at an optimal concentration of 10 ng/ml. To further substantiate the protective effect of GDF-15, we quantified neuron death by counting PI-stained (dead) cells. Fig. 1A (right panel) shows that GDF-15 significantly decreased cell death (4% TUNEL-positive cells). In the presence of IGF-1, an established anti-apoptotic molecule for CGN following K⁺ withdrawal (18, 23), cell death was reduced to 10%. GDF-15 reduced numbers of apoptotic neurons in low K⁺ to 15%. Together, these data indicate that GDF-15 is a potent neuroprotective factor for CGN that matches the effect of IGF-1, the best established protective factor in this culture system.

Effects of PI3K and MEK1/2 Inhibitors on GDF-15-induced Neuroprotection—To begin to analyze the signaling cascades activated by GDF-15, we chose specific inhibitors that inactivate core units of different signaling pathways. Inhibition of PI3K, which directs activation of Akt, has been shown to abolish protective effects of growth factors in CGN (24, 25). We used two selective inhibitors of PI3-kinase, wortmannin and LY294002, to block the activity of the enzyme in CGN. To determine whether GDF-15-induced survival is impaired by PI3K inhibitors, we first performed LDH measurements (Fig. 2A, left panel) and counted PI-stained cells (Fig. 2A, right panel). The protective effect of GDF-15 was significantly reduced in wortmannin (100 nM)- or LY294002 (50 µM)-pretreated cultures, indicating that PI3K activation is important for GDF-15 signaling. To examine the effect of inhibitors on DNA fragmentation, we performed TUNEL stainings. Fig. 2B shows an increased number of TUNEL-positive neurons in the presence of GDF-15 plus wortmannin compared with GDF-15 alone. However, GDF-15 protection was only partly (about 50%) attenuated by inactivation of the PI3K, suggesting that GDF-15 may employ additional mechanisms to protect CGN.

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Activation of extracellular signal-regulated protein kinases (ERKs) has been reported to contribute to neuronal cell survival in certain models of neurotoxicity (26–28). As our data indicated that GDF-15 is a potent protective factor for CGN, we hypothesized that regulation of ERK phosphorylation might be involved in GDF-15-induced survival. ERK1/2 activity is exclusively regulated through MEK1/2 phosphorylation, an immediate upstream dual-specificity kinase (29). We used U0126 (20 μM), which blocks MEK1/2 (25), and PD98059 (60 μM), a specific blocker of MEK1 (30), to inhibit ERK phosphorylation. Fig. 2A and B, show that inactivation of ERKs did not reverse the survival promoting effect of GDF-15. On the contrary, neuronal protection with GDF-15 was even more pronounced in the presence of U0126 (20 μM) or PD98059 (60 μM) as measured by LDH release and numbers of PI-stained cells. To address the question whether MEK inhibition alone is sufficient to promote CGN survival in low K⁺, we applied U0126 or PD98059 without GDF-15. Fig. 2A provides evidence that both inhibitors promoted survival, although U0126 showed a somewhat higher protection compared with PD98059. To examine whether the
MEK1/2 inhibitor increased cell viability by inhibiting DNA fragmentation, we performed TUNEL stainings and quantified the data. As shown in Fig. 2B, the MEK1/2 inhibitor U0126 significantly decreased numbers of TUNEL positive cells. These results suggest that low K⁺-induced apoptosis in CGN activates MEK1/2 during neuron death and that inhibition of this pathway clearly attenuates cell death.

**GDF-15 Induces Akt and GSK-3β Phosphorylation**—The serine/threonine protein kinase Akt is a downstream effector of PI3K and has been reported to play a critical role in promoting survival of a variety of different cell types (for reviews, see Refs. 31 and 32). As the PI3K inhibitor blocked GDF-15-induced CGN survival we investigated whether GDF-15 induces Akt phosphorylation in CGN cultured in low K⁺ (5K) conditions. As shown in Fig. 3A, GDF-15 significantly increased levels of phosphorylated Akt within 5 min after factor administration. This effect was still detectable after 24 h and decreased to basal level by 48 h (Fig. 3A). Akt activation was paralleled by an activation of Akt-mediated phosphorylation at Ser-21 of GSK-3β and Ser-9 of GSK-3β (33). We used an anti-phospho-GSK-3β (Ser-9) antibody to detect phosphorylated GSK-3β. As shown in Fig. 3A, GDF-15-induced phosphorylation of GSK-3β was paralleled by an activation of Akt. Moreover, preincubation of the cultures with the PI3K inhibitors wortmannin (100 nM) or LY294002 (10 μM) markedly blocked the GDF-15-induced Akt and GSK3 phosphorylation (Fig. 3B). Total Akt levels were not changed (Fig. 3A and B). These data corroborate our findings that GDF-15 rescues CGN from cell death via the PI3K/Akt survival pathway. Moreover, the rapid phosphorylation of Akt/GSK within 5 min after factor administration points to a direct effect of GDF-15.

**GDF-15 Blocks MEK/ERK Signaling in Low K⁺ CGN Cultures**—The neuroprotective effect of the MEK inhibitors U0126 and PD98059 on CGN maintained in low K⁺ alone suggests that ERKs may be activated in this system at a certain time point during apoptosis. To investigate this hypothesis, lysates obtained at various time points from low K⁺ CGN culture were subjected to Western blot analysis to detect phosphorylated (activated) ERK using an anti-phospho-ERK1/2 antibody. The same blot was stripped and reprobed for total Akt. BC, control lysate of cultures treated with purified supernatant of uninfected Sf9 cells. The quantification of the intensity was depicted below the respective blots.

**GDF-15 Prevents Cell Death by Regulating Akt and ERK Pathways**—The rapid phosphorylation of Akt/GSK within 5 min after factor administration points to a direct effect of GDF-15.
out affecting total ERK levels. Phosphorylation of MEK1/2 was also affected by GDF-15 (Fig. 6A). However, GDF-15 did not completely inhibit ERK activation, while U0126 did (cf. Fig. 4B). We hypothesized that this could be due to a complete suppression of the ERK cell death pathway by U0126 and an activation of the Akt survival pathway by GDF-15. To challenge this hypothesis we performed Western blot analysis using cell lysates isolated 24 h after GDF-15 (20 ng/ml) treatment in the presence and absence of U0126 (20 μM). Fig. 6B shows a complete suppression of ERK phosphorylation, while Akt remained phosphorylated in the presence of both GDF-15 and U0126.

**MEK1/2 Inhibitor and GDF-15 Block Phosphorylation of c-Jun**—The c-Jun protein and its phosphorylation have been shown to be involved in low K⁺-induced cell death (19, 21). Given that ERK can phosphorylate c-Jun in vitro (34), together with our observation that there is a slow and sustained ERK activation in low K⁺, we hypothesized that ERK could be a potential upstream regulator of c-Jun. We performed Western blots using cell lysates obtained from various time points after K⁺ deprivation and probed them with phospho-specific c-Jun and total c-Jun antibodies. As shown in Fig. 7A, c-Jun phosphorylation increased slightly at 3 h after K⁺ deprivation, yet both protein and phosphorylation increased robustly and in parallel to ERK activation at 6 h and remained high for 24 h. Evidence that ERK regulates c-Jun protein and phosphorylation in low K⁺-induced apoptosis was obtained from ERK inhibition studies. The potent inhibitor of MEK/ERK signaling, U0126, was added to low K⁺ cultures for 9, 12, and 24 h, and lysates were subjected to Western blot analysis. Fig. 7A shows that in the presence of U0126 (20 μM) c-Jun phosphorylation was dramatically reduced, and the protein level was markedly decreased. Interestingly, GDF-15, which attenuated ERK phosphorylation, also markedly reduced c-Jun phosphorylation and protein levels. (Fig. 7A).

The stress-activated kinases, JNK and p38, are known to regulate c-Jun gene expression and phosphorylation (35–37). In accordance with previous studies (21, 38), we observed a high activation of JNK and a low activation of p38 in low K⁺ that were not affected by U0126 or GDF-15 treatments (Fig.
Fig. 6. A, GDF-15 blocks low K⁺ (5K)-induced sustained ERK activation. GDF-15 was added to 5K cultures for the indicated time points, and the cells were lysed and processed for Western blot analysis to detect phosphorylated ERK (p-ERK). The same blot was stripped to detect total ERK (ERK). Similarly, the representative lysates were processed to detect phosphorylation of MEK1/2 (p-MEK). B, GDF-15- and U0126-treated low K⁺ (5K) cultures show active Akt. The cells in 5K were treated with GDF-15 (20 ng/ml) and/or U0126 (20 μM) for 24 h. The cell lysates were processed for Western blotting to detect phosphorylated ERK and ERK. The representative samples were processed to detect phosphorylation of Akt (p-Akt) —no treatment; BC, control lysate of cultures treated with purified supernatant of uninfected Sf9 cells. The quantification of the intensity was depicted below the respective blots.

7B). Thus, activation of ERK regulates c-Jun protein synthesis and phosphorylation in low K⁺ CGN cultures. Treatment with either U0126 or GDF-15 abolishes this signaling and protects neurons from cell death.

The Anti-apoptotic Effect of GDF-15 Inhibits ROS Formation—ROS generated by oxidative stress are known to be important cell death effector molecules in low K⁺ CGN cultures (20, 39). We first studied the time course of ROS generation in low K⁺ cultures using the fluorescent ROS indicator dye DCF-H₂. DCF-H₂ gets oxidized to DCF by ROS, which, upon excitation, emits fluorescence (40, 41). Fig. 8A shows two peaks in ROS formation at 6 and 12 h and return to base-line levels at 24 h. We next investigated ROS generation after 6 h in low K⁺ in the presence of GDF-15 (20 ng/ml). As shown in Fig. 8B, GDF-15 effectively prevented ROS generation. The MEK inhibitors (PD 98059 (60 μM) and U0126 (20 μM)) did not affect DCF fluorescence, corroborating the notion that ROS generation is upstream of ERK activation.

DISCUSSION

GDF-15 is a novel divergent member of the TGF-β superfamily with largely unknown functions. Recent studies demonstrated that the molecule is expressed in the central nervous system, and the first functional characterization of the factor revealed pronounced neurotrophic effects on midbrain dopaminergic and raphe serotonergic neurons in vitro and in vivo (15). However, it is not clear whether other central nervous system neuron populations are also promoted by GDF-15. TGF-β can affect neurons in both anti- and pro-apoptotic fashions. Thus, elimination of TGF-β in chick embryos has been shown to abolish ontogenetic neuron death (36, 42) acting as a master molecule in the regulation of pro-apoptotic signaling cascades. In contrast, TGF-β can also synergize with neurotrophic factors, e.g. neurotrophins, fibroblast growth factor-2, and GDNF, in the promotion of neuron survival (43–47). With regard to GDF-15, a pro-apoptotic role in several cell lines has been suggested (48–51). Moreover, our observation that GDF-15 is robustly induced in lesioned cortical neurons (16) can be interpreted in terms of both an anti- or pro-apoptotic role. To clarify this, we started to analyze the effect of GDF-15 and underlying mechanisms on neuronal apoptosis using the well established CGN model (18). Reverse transcriptase-PCR analysis showed that cultured granule neurons express GDF-15, but expression is down-regulated upon withdrawal of survival promoting signals (not shown). The present study demonstrates that GDF-15, in contrast to TGF-β2, which has been shown to accelerate apoptosis in CGN (52), prevents primary CGN from low K⁺-induced apoptosis. Concerning underlying mechanisms, we first showed that the protective effect of GDF-15 was attenuated by inhibitors of the Akt-activating PI3K. Western blot analyses clearly confirmed the functional results and demonstrated a GDF-15-dependent activation of Akt and its downstream target GSK-3β. Consistent with our data is the observation that other TGF-β members, including GDNF, neurturin, persephin, and TGF-β1, promote cell survival via the PI3K/Akt pathway in various cell types (53–57).

We next analyzed whether the MEK/ERK pathway may be involved in GDF-15-dependent neuron rescue. Surprisingly, we found that GDF-15-induced protection in CGN was even more pronounced when ERKs were inactivated via selective inhibition of MEK. Moreover, MEK inactivation alone led to a potent protection against low K⁺-induced apoptosis of CGN. We observed slow and sustained ERK activation in CGN undergoing low K⁺-induced cell death. Down-regulation of ERK by MEK inhibitors led to a prevention of low K⁺-induced apoptosis. These findings corroborate several recent reports showing a pro-apoptotic involvement of ERK in neuronal cell death (58, 59). Moreover, recent studies also demonstrate that 1,2-chloropropionate- and kainate-mediated cell death in CGN (60, 61) is attenuated by MEK inhibition. Most importantly, GDF-15 clearly suppressed the persistent ERK activity in CGN. Prevention of cell death by MEK inhibitors is unexpected, because typically the ERK pathway has been credited with proliferative and cell survival responses (62, 63). However, our results together with the above studies expand the functional role of ERKs and suggest that the duration of ERK activation might determine pro- or anti-apoptotic decision of the cell.

The AP-1 transcription factor family members c-fos and c-jun have been implicated with pro-apoptotic functions (see Ref. 64 for a review). The role of c-Fos in low K⁺-induced CGN cell death is not known. A requirement of c-Jun activation in mediating low K⁺-induced CGN apoptosis has previously been demonstrated by Watson et al. (21). The present study has
and 24 h. (59) and the densitometric quantification of pJNK levels for 9, 12, and the phosphorylated JNK (p-JNK) levels for 9, 12, and 24 h. ERK (p-ERK) and the densitometric quantification of p-c-Jun and c-Jun (p-c-Jun), and phosphorylated c-Jun (p-p38) levels for 9, 12, and 24 h. After switching to low K⁺/H9262 were treated with U0126 (20 μM) or GDF-15, which may further provide evidence that c-Jun phosphorylation and protein levels increase in parallel to the increase in ERK activation. Notably, MEK1/2 inhibition or treatment with GDF-15, which both inhibit ERK activation, also inhibited c-Jun protein up-regulation and phosphorylation. Although the in vivo role of ERK in regulating c-Jun has yet to be determined (65), evidence that c-Jun can be phosphorylated by ERK in vitro (34) and our finding that MEK1/2 inhibition attenuates the increase in c-Jun protein and phosphorylation argue in favor of the possibility that ERK may be directly involved in regulating c-Jun activity. GDF-15-induced inhibition of c-Jun indicates that the factor prevents cell death by down-regulating a key signal in the mediation of cell death.

Whether GDF-15-mediated inhibition of c-Jun transmits to other important regulators of cell death remains open. Possible target molecules with roles in cell cycle control and apoptosis are cell cycle-regulated protein kinases (67). Several studies have demonstrated a MEK/ERK-mediated regulation of the cyclin-dependent kinases (CDKs) cyclin D1, cyclin E, and cyclin kinase inhibitors p21 and p27. Activation of CDKs and cyclin kinase inhibitors can result in cell cycle arrest or apoptosis (68–70). In the nervous system CDKs have been shown to be involved in the death of PC12 cells and postmitotic neurons (71–74). For example, serum-deprived neuronal PC12 cells and sympathetic neurons deprived of trophic support contain inappropriate amounts of cyclin B and cyclin D transcripts prior to cell death (75, 76). Most importantly, Padmanabhan et al. (77) documented the requirement of CDK proteins in low K⁺-induced CGN cell death. They demonstrated an up-regulation of cyclin D1 and cyclin E after 6 h in low K⁺. This is consistent with the late and persistent ERK induction observed in our studies. Although the mechanisms underlying CDK-activated neuronal cell death are still unknown, non-neuronal cells have provided model systems for the control of cell death via the MEK/ERK signaling pathway. The ability of MEK inhibitors to regulate CDKs and cyclin kinase inhibitor proteins (70, 78, 79), the presence of a consensus AP-1 promoter sequence in cyclin D1, and its direct induction by c-Jun (67) suggest that the strong MEK/ERK/c-Jun signals observed in this study may direct the regulation of cell cycle protein kinases for the orchestration of neuronal cell death.

To elucidate the GDF-15-mediated mechanisms that suppress ERK activation in CGN, we continued by documenting the possible role of ERK activation in modulating c-Jun activity. GDF-15-induced inhibition of c-Jun indicates that the factor prevents cell death by down-regulating a key signal in the mediation of cell death.

The time course of ROS production in low K⁺ as detected by DCF fluorescence was measured as described under “Experimental Procedures.” B, effect of GDF-15 and MEK inhibitors on ROS generation. After switching the culture to low K⁺ (5K) medium, GDF-15 (20 ng/ml), U0126 (20 μM), or PD98059 (60 μM) were added and DCF fluorescence was measured after 6 h as described under “Experimental Procedures.” *, p < 0.05 compared with 5K cultures.
duces ERK activation (80–82). We now show for the first time that GDF-15 inhibits the formation of ROS. Furthermore, the inability of both MEK inhibitors to prevent ROS generation suggests that ROS generation is upstream of ERK activation in low K⁺ CGN. It should be noted, however, that in other systems, as superior cervical ganglionic neurons, nerve growth factor can prevent ROS generation by activating the ERK pathway (83), suggesting that ROS formation in this system is downstream of ERK.

The present in vitro work has provided clues for the possible signaling pathways that are activated by GDF-15 for executing its functions. Activation of Akt is known to play a critical role in controlling the balance between survival and apoptosis (84, 85). The discovery that ERK inhibition promotes survival was surprising but needs to be discussed in the context of the putative in vivo relevance of this finding. Recent in vivo studies have revealed that inhibition of ERK can reduce infarct volume after focal cerebral ischemia, suggesting a deleterious effect of ERK activation (86, 87). In addition, an abnormal punctate staining pattern of activated ERK has been described in the brains of patients with Alzheimer’s disease (88). While the status of ERK activation in patients with Parkinson’s disease is currently unknown, the involvement of ERK in 6-hydroxydopamine toxicity (89), and our previous in vitro studies, in which we demonstrated potent protective effects of GDF-15 in 6-hydroxydopamine-lesioned rat brains (15), suggest that the GDF-15 may exert its protective effect in neurons through an ERK-inactivating mechanism.

In conclusion, we have demonstrated that GDF-15 prevents cell death in low K⁺ CGN cultures by two different modes of action. First, GDF-15 induces survival by activating Akt and GSK via the PI3K. Second, GDF-15 down-regulates the MEK/ERK/Jun cell death signaling pathway. Moreover, GDF-15 also attenuates ROS formation, an inducer of ERK, thereby preventing cell death. Putative cross-talks between these pathways need to be addressed by future experiments. An important issue also to be resolved in the future concerns the identity of GDF-15 receptors. Finally, the question whether GDF-15 may act as a regulator of apoptosis for CGN in vivo remains to be answered. Ongoing studies with a GDF-15-deficient lacZ knock-in transgenic mouse, recently developed in our laboratory, may help to address this question.

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