PIGMENT EPITHELIUM-DERIVED FACTOR INDUCES PRO-SURVIVAL GENES THROUGH CYCLIC AMP-RESPONSIVE ELEMENT BINDING PROTEIN AND NUCLEAR FACTOR KAPPA B ACTIVATION IN RAT CULTURED CEREBELLAR GRANULE CELLS: IMPLICATION FOR ITS NEUROPROTECTIVE EFFECT

T. YABE,a,b,d K. KANEMITSU,a,b T. SANAGI,a,b J. P. SCHWARTZc AND H. YAMADAa,b,d

aKitasato Institute for Life Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo 108-8641, Japan
bGraduate School of Infection Control Sciences, Kitasato University, Tokyo 108-8641, Japan
cNeurotrophic Factors Section, NINDS, National Institutes of Health, Bethesda, MD 20892-4126, USA
dOriental Medicine Research Center, The Kitasato Institute, Tokyo, Japan

Abstract—Pigment epithelium-derived factor (PEDF) protects immature cerebellar granule cell neurons (CGCs) against apoptosis induced by K+ and serum deprivation. However, the precise mechanism of this protection remains unknown. We recently reported that the transcription factor nuclear factor kappa B (NF-κB) is activated in PEDF-treated CGCs. Although it is well known that NF-κB blocks apoptotic cell death through the induction of pro-survival factors, the effects of PEDF on the expression of these factors are not fully understood. In this study, we employed the use of reverse transcriptase–polymerase chain reaction to analyze the gene expression of certain pro-survival genes and found that genes such as c-IAP1, c-IAP2, FLIPs, A1/Bfl-1 and Mn-SOD were induced in PEDF-treated neurons. On the other hand, no induction was observed of the pro-apoptotic Bcl-2 family members Bax and Bid at any time from 3 to 24 h following PEDF addition. Furthermore, phosphorylation of cyclic AMP-responsive element binding protein (CREB) and increment of nuclear cyclic AMP-response element (CRE)‐like DNA binding were observed in PEDF-treated CGCs. The anti-apoptotic effect of PEDF was blocked by overexpression of dominant negative CREB or a mutated form of IκBα. These results suggested that induction of both CRE- and NF-κB-dependent genes is required for the observed neuroprotective effects of PEDF on CGCs. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: pigment epithelium-derived factor, PEDF, pro-survival genes, neuronal apoptosis, transcription factors, signal transduction pathway.

Pigment epithelium-derived factor (PEDF) is a 50-kDa glycoprotein and a member of the serine protease inhibitor (SERPIN) gene family (Tombran-Tink et al., 1991). Expression of PEDF mRNA was detected in a broad range of human fetal and adult tissues, including almost all areas of the brain (Tombran-Tink et al., 1996). It is neurotrophic and protective for neurons cultured from the cerebellum, hippocampus, spinal cord (motor neurons), and retina (Tombran-Tink and Barnstable, 2003). Unlike other SERPINs, PEDF possesses no inhibitory activity against any of the known proteases, and its neuroprotective effect does not appear to require the serpin-reactive loop located toward the carboxy end of the polypeptide (Becerra et al., 1995). Although PEDF appears to play an important role in the survival and maintenance of various kinds of neurons, the precise mechanism by which PEDF acts on cerebellar granule cells (CGCs), or any other neuron type, remains unknown.

Cultured CGCs have been widely used in investigations concerning the signaling pathways used for neuronal survival or apoptosis. In the presence of serum, CGCs must acquire the appropriate level of depolarization, achieved by inclusion of a high concentration (25 mM) of extracellular K+ (Gallo et al., 1987). Lowering the K+ concentration to physiological levels (5 mM) (D’Mello et al., 1993) or removing the serum (Atabay et al., 1996) induced apoptosis in CGCs. We previously demonstrated that PEDF most effectively blocked low K+(LK)-induced apoptosis when added 24 h prior to the induction of apoptosis, and provided some protection when added simultaneously (Araki et al., 1998). Furthermore, PEDF induced an increase in the phosphorylation of IκBα, decreased the level of IκB proteins, and induced the translocation of RelA (p50) protein to the nucleus followed by a time-dependent increase in nuclear factor kappa B (NF-κB)–DNA binding activity in cultured CGCs (Yabe et al., 2001). These observations raise the possibility that specific changes in gene expression via the activation of signal transduction pathways and transcription factors are required for the anti-apoptotic effect of PEDF. In this study, the intracellular mechanisms by which PEDF protects CGCs against apoptosis were explored, with particular attention being paid to the role of NF-κB, cyclic AMP-responsive element bind-
ing protein (CREB) and serine/threonine protein kinase Akt protein. Our data indicated that the induction of pro-survival genes via NF-κB and CREB activation is involved in the anti-apoptotic effects of PEDF on CGCs.

**EXPERIMENTAL PROCEDURES**

**Materials**

Antibodies were obtained from the following sources: anti-phospho-specific CREB and CREB antibodies from Upstate Biotechnology (Lake Placid, NY, USA); anti-phospho-specific Akt, anti-Akt, anti-phospho-specific extracellular signal-regulated kinase (Erk)(J)/2, and anti-Erk1/J/2 antibodies from Cell Signaling Technology (Beverly, MA, USA). All culture reagents were obtained from (St. Louis, MO, USA). The Reverse Transcription System and the CellTiter 96® AQueousNon-Radioactive Cell Proliferation Assay were purchased from Promega (Madison, WI, USA) and anti-Erk1/J/2 antibodies from Cell Signaling Technology (Beverly, MA, USA). All culture reagents were obtained from (St. Louis, MO, USA). The ViraPower™ Adenoviral Expression System was obtained from Invitrogen Corporation (Carlsbad, CA, USA).

**Recombinant human PEDF**

The PEDF used in this study was prepared as previously described (Becerra et al., 1993). Briefly, a truncated recombinant expression construct (Asp44-Pro418) derived from human PEDF cDNA was expressed in *Escherichia coli*, purified from the bacterial inclusion bodies, and stored in urea buffer (50 mM Na phosphate, 150 mM NaCl, pH 6.5, containing 4 M urea). An equivalent volume of urea buffer was added to all control/untreated samples. Analysis of recombinant PEDF in terms of endotoxin lipopolysaccharide content demonstrated undetectable levels (<0.05 endotoxin units/ml) at the lowest dilution of recombinant PEDF used in assays) using the *limulus* amebocyte lysate method (Associates of Cape Cod, Falmouth, MA, USA).

**Primary CGCs culture**

CGCs were prepared from 8-day-old Wistar rat pups (Japan SLC, Shizuoka, Japan) as previously described (Taniwaki et al., 1995). All experimental protocols were approved by the Experimental Animal Committee of the Kitasato University and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23; revised 1996). Every effort was made to minimize the number of animals used and any pain or discomfort. Cells were plated in serum-containing medium (Eagle’s basal medium (BME) with 25 mM KCl, 2 mM glutamine, 0.5% (v/v) penicillin/streptomycin and 25 mM KCl, 2 mM glutamine, 0.5% (v/v) penicillin/streptomycin and 10% heat-inactivated fetal bovine serum) in poly-D-lysine-coated six well plates, eight well chamber slides, or 96 well plates at 3.0×10^6 cells/cm² and grown at 37 °C in a humidified atmosphere. Cytosine arabinoside was added on day 1 *in vitro* (DIV1) at a final concentration of 10 μM. On DIV2, the CGC cultures consisted of >98% neurons (stained with anti-neuron-specific enolase antibody, <1% astrocytes (stained with anti-glial fibrillary acidic protein antibody) and <1% microglia (stained with anti-OX42 antibody).

**Reverse transcriptase–polymerase chain reaction (RT-PCR)**

Total RNA was extracted from CGCs according to the manufacturer’s instructions for TRIZOL Reagent (Invitrogen). One microgram of total RNA was converted to first strand cDNA using the First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The resulting cDNA was subjected to PCR analysis. Cyclophilin was used as the non-changing control RNA. The nucleotide sequences of the specific primers used are summarized in Table 1.

**Western blot analysis**

Activation of signal transduction pathway was determined by immunoblotting as described previously (Yabe et al., 2005). In brief, neurons treated with rhPEDF were washed with cold PBS, solubilized with SDS sample buffer, and then sonicated for 10 s. The lysates were boiled for 5 min and centrifuged for 15 min. The supernatants were subjected to SDS-PAGE. Equal amounts of lysate protein were run on a 10% SDS polyacrylamide gel and transferred to nitrocellulose membranes. Nitrocellulose blots were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Triton X-100 (TBST), and then incubated with primary antibodies [anti-phospho-specific Akt, 1:2000; anti-Akt, 1:2000; anti-phospho-specific CREB, 1:2000; anti-CREB, 1:2000; anti-phospho-specific Erk1/J/2, 1:2000; anti-Erk1/J/2, 1:2000] in TBST containing 3% BSA. After washing, the blots were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG at a dilution of 1:5000. Immunoreactive phosphate-CREB and total CREB proteins were detected with the enhanced chemiluminescent protocol (Amersham Pharmacia Biotech).

**Nuclear extract preparation and electrophoresis mobility shift assay (EMSA)**

Nuclear extracts for the EMSA were prepared by a mini-extraction protocol (Schreiber et al., 1989). EMSA was performed with a commercial kit (Promega) according to the manufacturer’s instructions. Two micrograms or 5 μg of nuclear extract was incubated with a 32P-labeled DNA sequence containing the cyclic AMP response element (CRE)-like consensus sequences. The DNA–

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-IAP1</td>
<td>CCAGCTGGCCCTGCAACCTCTT</td>
<td>GGTCATCTCCGGGTTCCCAAC</td>
</tr>
<tr>
<td>c-IAP2</td>
<td>ACATTCTCCCCAGCTGCCATTC</td>
<td>CTCTGTCCTCGCTCTCTTCT</td>
</tr>
<tr>
<td>FLIPs</td>
<td>GCCAAAGGAAAGATTTCTTGG</td>
<td>AGCATTTTCCAGTACTGG</td>
</tr>
<tr>
<td>A1/Bfl-1</td>
<td>ATCCATCTCCCGAGGACAAT</td>
<td>ACATCCAGGCCAATCTCGTCTT</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>GACCTGCGCTTAGCAGATATGG</td>
<td>GACCTGCTCTTTATTGGAGC</td>
</tr>
<tr>
<td>Bid</td>
<td>AGGTGGCTCTTCCTCTCGTCTC</td>
<td>CTTTGCTCTATGGCCCTGACCTT</td>
</tr>
<tr>
<td>Bax</td>
<td>TTTGCGCTTTTACTCGTGCGCATC</td>
<td>GCCAGGGTCTCTGGAATTTCCC</td>
</tr>
<tr>
<td>IκBα</td>
<td>CACTCTCTTCTCGTTCGGAAC</td>
<td>CACACTCAACGAGGATGACACAGC</td>
</tr>
<tr>
<td>dIAPs</td>
<td>CCGGAGGCTTACATAACCTC</td>
<td>GAGGACCCATTAAAACTCCAGGG</td>
</tr>
<tr>
<td>cyclin D1</td>
<td>AGATGATGGTGTAGTTGATAC</td>
<td>CACAAGATGTCGCTGGTCG</td>
</tr>
</tbody>
</table>

Table 1. Primers employed in RT-PCR analysis
protein complexes were separated from unbound oligonucleotide by electrophoresis through native 6% polyacrylamide gels using 0.5× TBE buffer. Following electrophoresis, the gels were dried and analyzed using a phosphorimager (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Adenovirus constructs and transfection

Replication-defective adenovirus vectors expressing a mutated nondegradable IκBα, with serines 32 and 36 mutated to alanines (Adeno-IκBα(M) under the control of the cytomegalovirus (CMV) promoter, were constructed using the ViralPower™ Adenoviral Expression System (Invitrogen) as previously described (Yabe et al., 2005). A recombinant adenovirus vector expressing a mutant of Akt (Ad.dnAKT) in which the phosphorylation sites at Ser133 and Thr308 were changed to alanines was a gift from Dr. Kenneth Walsh (Boston University School of Medicine, Boston, MA, USA). Adenoviral vectors were amplified in 293A cells and purified by ultracentrifugation through a CsCl gradient. Determination of infectious titer was performed using the plaque assay on 293A cells. Recombinant adenovirus expressing dnAKT, dnCREB, IκBα(M) under the control of the cytomegalovirus (CMV) promoter, were constructed using the ViralPower™ Adenoviral Expression System (Invitrogen) as previously described (Yabe et al., 2005). A recombinant adenovirus vector expressing a mutant of Akt (Ad.dnAKT) in which the phosphorylation sites at Ser133 and Thr308 were changed to alanines was a gift from Dr. Kenneth Walsh (Boston University School of Medicine, Boston, MA, USA).

Cell survival assays

CGCs were infected with recombinant adenoviruses (m.o.i. = 10) at the time of plating and treated with or without 20 nM PEDF at DIV2. One day following PEDF treatment, the medium was changed to 5 mM KCl/serum-free apoptosis medium for 24 h. On DIV4, cell survival was assessed using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega). Briefly, CGCs were incubated in a CO2 incubator for 1 h with MTS (3-(4,5-

Statistical analysis

For statistical analysis, data were compared by one-way analysis of variance and Fisher’s protected least square difference.

RESULTS

PEDF induces pro-survival genes in CGCs

We recently reported that NF-κB was activated in PEDF-treated CGCs (Yabe et al., 2001). Recent studies demonstrated that activation of the transcription factor NF-κB plays a crucial role in preventing neuronal apoptosis by promoting the expression of pro-survival genes (Mattson et al., 2000; Mattson and Camandola, 2001; Denk et al., 2000). Potential pro-survival genes regulated by NF-κB include those that code for inhibitor-of-apoptosis proteins (IAPs) (Chu et al., 1997; Wang et al., 1998). Bcl-2 family members (Glasgow et al., 2000; Zong et al., 1999), manganese superoxide dismutase (Mn-SOD) (Darville et al., 2000) and FLICE inhibitor protein (FLIP) (Michau et al., 2001). We therefore examined the effect of PEDF on the expression of pro-survival gene mRNAs in CGCs using RT-PCR analysis. Consistent with our previous study (Yabe et al., 2001), the level of Mn-SOD mRNA was up-regulated following PEDF treatment (Fig. 1). Furthermore, PEDF treatment also resulted in a temporal increase in c-IAP1, c-IAP2, FLIPs and A1/Bfl-1 mRNA levels in CGCs, whereas the mRNA levels of pro-apoptotic genes such as Bax and Bid remained unchanged.

Effect of PEDF on the down-regulation of pro-survival genes by LK

CGCs undergo apoptosis when they are switched from medium containing serum and 25 mM KCl (HK/serum+) to serum-free medium containing 5 mM KCl (LK/serum−) (D’Mello et al., 1993; Yan et al., 1994; Galli et al., 1995). Piccioli et al. (2001) reported that switching from HK to LK reduced NF-κB nuclear levels in CGCs, and suggested that this reduction was involved in the induction of CGC apoptosis. Given this, we then set out to determine whether changes in the level of pro-survival gene mRNAs was involved in LK/serum− treatment, whereas Bid and Bax mRNA levels remained unchanged under the same conditions. Interestingly, the reduction of c-IAP2, FLIP, Mn-SOD and A1/Bfl-1mRNA levels in LK/serum−-treated cells was markedly reversed following pretreatment with PEDF for 24 h (Fig. 2).

PEDF induces phosphorylation of CREB in HK and LK conditions

The signal transduction pathways that mediate the cell survival promoting actions of neurotrophic factors and growth factors are currently being worked out, and in many cases are associated with the phosphorylation of Akt, mitogen-activated protein kinase (MAPK) and CREB (Patapoutian and Reichardt, 2001; Riccio et al., 1999).

**Fig. 1.** RT-PCR analysis of survival-related genes. CGCs at DIV2 were treated with PEDF (20 nM) for the indicated times. Total RNA was extracted, reverse-transcribed, and subsequently subjected to PCR using specific primers. Cyclophilin served as the unchanging control mRNA. Three to four different RNA samples were analyzed for each specific mRNA shown.
In an effort to determine whether these factors play a role in the anti-apoptotic effect of PEDF, the phosphorylation of these proteins was evaluated following PEDF treatment. The phosphorylation of CREB and Akt rapidly increased in PEDF-treated CGCs compared with untreated cells (Fig. 3A and B). In contrast, the phosphorylation of Erk1/2, p38MAPK and c-Jun NH2-terminal kinase remained unchanged following PEDF treatment (Fig. 3C and unpublished results). Furthermore, EMSA revealed that PEDF treatment led to a transient increase in nuclear CRE-DNA binding activity at 0.5 h in CGCs.

In order to investigate the signal transduction pathways involved in the apoptosis of CGCs, the phosphorylation of Erk1/2, Akt and CREB was examined after switching to LK/serum(-) cultures. Total RNA was extracted and RT-PCR was performed. Cyclophilin served as the unchanging control mRNA. Two to four different RNA samples were analyzed for each specific mRNA shown.

![Fig. 2. PEDF induces survival-related genes in LK/serum(-) cultures. CGCs at DIV1 were treated with or without PEDF (20 nM) for 24 h, and then cultures were switched to HK or LK medium. After 6 h in culture, total RNA was extracted and RT-PCR was performed. Cyclophilin served as the unchanging control mRNA. Two to four different RNA samples were analyzed for each specific mRNA shown.](Image)

(Fig. 4A). And higher ERK phosphorylation in PEDF treated cells was observed at 24 h after LK/serum− treatment (Fig. 4B).

**Inactivation of NF-κB blocks the anti-apoptotic effect and induction of pro-survival genes by PEDF**

We previously reported that pretreatment with the proteasome inhibitor N-acetyl-Leu-Leu-norleucinal (ALLN) blocked the ability of PEDF to activate NF-κB and to protect neurons against LK/serum(−)-induced apoptosis (Yabe et al., 2001). The proteasome is a multicatalytic protease complex responsible for the degradation of most intracellular proteins, including proteins crucial to apoptosis (Voorhees et al., 2003). Thus, we could not exclude the possibility that degradation of intermediates besides IκB protein was involved in ALLN inhibition of the neuroprotective effect of PEDF. In order to provide further evidence that NF-κB activation is crucial to the anti-apoptotic effect of PEDF, CGCs were infected with an adenovirus vector expressing a mutated non-degradable IκBα (IκBαM) or with a control virus (Ad.LacZ), and treated with PEDF and LK/serum(−). In LK/serum(−)-treated cultures, overexpression of IκBαM almost completely inhibited the protective effect of PEDF as assessed by the MTS assay, whereas the control virus had no such effect (Fig. 5A). Furthermore, the expression of IκBαM almost completely abolished the induction of c-IAP1, c-IAP2, FLIPs, A1/Bfl-1 and Mn-SOD mRNA levels following PEDF treatment (Fig. 5B).

**Inactivation of CREB blocks the anti-apoptotic effect and induction of pro-survival genes by PEDF**

In an effort to ascertain the functional significance of PEDF-induced activation of CREB in CGCs, cells were infected with an adenovirus expressing a dominant negative mutant of CREB in which the phosphorylation site at Ser133 was changed to alanine (Ad dnCREB) or a control virus (Ad.LacZ), and subsequently treated with PEDF and LK/serum(−). CGCs infected with Ad dnCREB strongly expressed dnCREB mRNA while those infected with Ad.LacZ expressed LacZ mRNA (Fig. 6B). As shown in Fig. 6A, overexpression of dnCREB augmented LK/serum(−)-induced apoptosis and completely inhibited the anti-apoptotic effect of PEDF. Furthermore, the expression of dnCREB almost completely abolished the induction of c-IAP1, c-IAP2, FLIPs, A1/Bfl-1 and Mn-SOD mRNA levels following PEDF treatment (Fig. 6B).

Although overexpression of the dominant negative mutant Akt partially attenuated the induction of pro-survival genes by PEDF (Fig. 7B), Akt inhibition did not affect PEDF protection against LK/serum(−)-induced apoptosis (Fig. 7A).

**DISCUSSION**

The balance between pro-apoptotic and pro-survival molecules can determine cellular fate. Activation of the family of caspases is a key event in the induction of apoptosis in most mammalian cells (Zimmermann and Green, 2001). Caspase-induced apoptosis is regulated by endogenous...
antagonists such as FLIP that prevent processing (Krueger et al., 2001), and anti-apoptotic proteins of the IAPs family that block function (Hay, 2000). The release of intermediates such as cytochrome-C from the mitochondrial intermembrane space that occurs as an initial step is blocked by anti-apoptotic members of the Bcl-2 family proteins (Burlacu, 2003). The anti-oxidant enzyme Mn-SOD blocks or delays oxidative stress-related changes in the mitochondrial membrane potential, and the subsequent release of cytochrome-C and induction of apoptosis (Martin-Romero et al., 2002). In this paper, we demonstrated that PEDF induced pro-survival genes such as c-IAP1, c-IAP2, FLIPs, A1/Bfl-1 and Mn-SOD. Potential NF-κB binding sites have been identified in the 5′ flanking region of all the aforementioned genes (Chu et al., 1997; Wang et al., 1998; Zong et al., 1999; Darville et al., 2000). Overexpression of any of these genes inhibits cell death induced by various apoptotic stimuli (Guo et al., 1999; Simons et al., 1999; Suhara et al., 2001; D’Sa-Eipper et al., 2002). In contrast, no induction of the pro-apoptotic Bcl-2 family members Bax and Bid was observed in PEDF-treated neurons at any time from 3 to 24 h following PEDF addition. These observations suggested that PEDF may influence the balance between pro-survival and pro-apoptotic signals in CGCs.

NF-κB is composed of homo- and heterodimers of members of the Rel family of related transcription factors that control the expression of numerous genes. Recent studies demonstrated that activation of NF-κB plays a critical role in preventing neuronal death in a number of models (Mattson et al., 2000; Mattson and Camandola, 2001). Moreover, inhibition of NF-κB by overexpression of IκB or treatment with proteasome inhibitors promoted apoptosis in neurons (Choi et al., 2000; Pasquini et al., 2000). These observations suggested that NF-κB plays an important role in neuronal survival and death. Although the precise mechanism by which NF-κB regulates neuronal survival or death is currently under intense investigation, the transcriptional activation of pro-survival genes is considered the most common way for NF-κB to antagonize apoptosis (Barkett and Gilmore, 1999). We demonstrated here that adenoviral gene transfer of IκBαM completely inhibited PEDF-induced expression of pro-survival genes such as c-IAP1, c-IAP2, FLIP, A1/Bfl-1 and Mn-SOD, suggesting that PEDF up-regulates pro-survival genes through activation of NF-κB. Furthermore, PEDF protection against LK/serum(−)−induced apoptosis was blocked by overexpression of IκBαM. These observations suggested that NF-κB-dependent gene expression is required for the anti-apoptotic effect of PEDF on CGCs.
The CRE has been implicated in the regulation of the expression of many genes and cellular processes important in neuronal function. CRE sites exist in the promoter regions of several pro-survival genes such as c-IAP1, c-IAP2, Bcl-2, Mn-SOD, and neurotrophins (Dong et al., 2002; Nishihara et al., 2003; Kim et al., 1999; Wilson et al., 1996; Timmusk et al., 1993). Members of CREB/ATF (activation of transcription factor) bind to CREs within promoter and enhancer sequences of many genes and are activated by phosphorylation on specific serine residue. CREB is a 43 kDa nuclear transcription factor that was originally found to be activated by cAMP-dependent protein kinase. Recent studies demonstrated that phosphorylation of CREB is also mediated by Erk, p38 MAPK, calcium–calmodulin kinase (CaMK) and the Akt protein kinase pathway (Lonze and Ginty, 2002). A constitutive binding of CREB to CRE consensus sequences in the absence of stimulus has been reported (Quinn and Granner, 1990), where phosphorylation of CREB results in the enhancement of transcriptional activation by the recruitment of additional coactivators (Chrivitaka et al., 1993; Quinn, 1993). Alternatively, phosphorylation of CREB might promote binding to the CRE (Yamamoto et al., 1988; Nichols et al., 1992). In this paper, we demonstrated that PEDF induced the phosphorylation of CREB under both normal (HK/serum+) and apoptotic (LK/serum−) conditions. We also demonstrated that the nuclear CRE-like DNA binding activity was induced in PEDF-treated neurons. The ability of PEDF to enhance its binding activity, without inducing any change in overall CREB levels, suggested that binding of CREB to CRE consensus sequences could be regulated by phosphorylation in CGCs. Furthermore, the overexpression of dominant negative CREB almost completely blocked the anti-apoptotic effect, in addition to the expression of c-IAP1, c-IAP2, FLIPs, A1/Bfl-1 and Mn-SOD mRNA induced by PEDF. These observations suggested that CRE-dependent gene expression, in addition to NF-κB activation, is required for the anti-apoptotic effect of PEDF on CGCs. Whether the function of

**Fig. 4.** PEDF induces phosphorylation of CREB in LK/serum(−) cultures. CGCs at DIV1 were treated with or without PEDF (20 nM) for 24 h, and then cells were switched to serum-free medium with LK. After switching the medium at the indicated time points, lysates were prepared and analyzed by Western blotting using antibodies against phosphorylated Akt (p-Akt), Akt, phosphorylated CREB (p-CREB), CREB, phosphorylated Erk1/2 (P-Erk1/2) or Erk1/2. Results are representative of three independent experiments.
other CREB/ATF family members and the interaction between NF-κB and CREB are involved in anti-apoptotic effect of PEDF will require further analysis.

The serine–threonine protein kinase Akt is a multifunctional regulator of cell survival, growth, and glucose metabolism (Datta et al., 1999). Akt possesses an N-terminal pleckstrin homology domain that binds phosphorylated lipids at the membrane in response to phosphatidylinositol 3-kinase (PI3-K) activation (Franke et al., 1995). Activation of the PI3-K/Akt pathway has a potent anti-apoptotic effect through (a) preventing the release of cytochrome c from mitochondria, (b) phosphorylating and thereby preventing activation of the pro-apoptotic Bcl-2 family member Bad, (c) increasing transcription of the anti-apoptotic genes through activation of transcriptional regulatory factors such as NF-κB, CREB and Forkhead, and (d) inhibiting the death protease caspase-9 (Brunet et al., 2001). Given that
The results of our study suggested that the protective up-regulation of anti-apoptotic gene expression by PEDF. Activation was involved in the anti-apoptotic effect and the precise mechanism associated with the anti-apoptotic effect of PEDF, identification of CRE- and NF-xB-dependent genes regulated by PEDF may help to understand the regulation of neuronal survival and to design new therapeutic strategies for neurodegenerative disorders.

In conclusion, we report here that NF-xB- and CRE-dependent gene expression is required for PEDF protection against LK/serum(-)-induced apoptosis. Although the precise mechanism by which PEDF activates NF-xB and CREB remains unclear, the ability to regulate the expression of pro-survival molecules such as IAPs, A1/Bfl-1, FLIPs and Mn-SOD would account for the potent anti-apoptotic effect of PEDF on LK/serum(-)-induced apoptosis. Although further work is required in an effort to delineate the precise mechanism associated with the anti-apoptotic effect of PEDF, identification of CRE- and NF-xB-dependent genes regulated by PEDF may help to understand the regulation of neuronal survival and to design new therapeutic strategies for neurodegenerative disorders.

Acknowledgments—We thank Dr. Patricia Becerra (NEI, NIH) for the gift of recombinant human PEDF and Dr. Kenneth Walsh (Boston University School of Medicine, Boston, MA, USA) for the gift of pAd.dnAKT. This work was supported in part by a Research Grant from Uehara Memorial Foundation, by a Research Grant for Young Researchers from Kitasato University, and by a Grant of the 21st Century COE Program, Ministry of Education, Culture, Sports, Science and Technology (MEXT).

REFERENCES


The phosphorylation of Akt was observed in PEDF-treated CGCs (Fig. 3), we set out to determine whether Akt activation was involved in the anti-apoptotic effect and the up-regulation of anti-apoptotic gene expression by PEDF. The results of our study suggested that the protective effect of PEDF against LK/serum(-)-induced apoptosis was not mediated via the activation of Akt, since inhibition of Akt by adenoviral transduction of dominant negative Akt did not prevent the anti-apoptotic PEDF effect or up-regulation of anti-apoptotic gene expression.

Fig. 7. Inactivation of Akt does not affect the anti-apoptotic effect of PEDF. (A) CGCs were infected (m.o.i. = 10) with Ad.dnAKT or Ad.LacZ at the time of plating and treated with or without 20 nM PEDF at DIV2. One day following PEDF treatment, the medium was changed to LK or HK. Cell viability was measured using the MTS assay after 24 h. Data represent mean ± S.D. (N=6) relative to untreated cultures. (B) CGCs were infected (m.o.i. = 10) with Ad.dnAKT (B) or Ad.LacZ (A and B) at the time of plating and treated with 20 nM PEDF at DIV 2. Following 6 h incubation with PEDF, total RNA was extracted and RT-PCR was performed as described in Experimental Procedures. Two to four different RNA samples were analyzed for each specific mRNA shown.


(Accepted 9 March 2005)