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KMUP-1 attenuates serum deprivation-induced neurotoxicity in SH-SY5Y cells: Roles of PKG, PI3K/Akt and Bcl-2/Bax pathways

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ABSTRACT

Aging populations with neurodegenerative disorders will gradually become a greater problem for society. Serum deprivation-induced cell death is recognized as one of the standard models for the study of neurotoxicity. Increasing evidence indicates that cGMP/PKG pathway may play a rescue role in serum deprivation-induced toxicity. The aim of this study was to investigate protective effects of KMUP-1, an enhancer of cGMP/PKG signaling on serum deprivation-induced neurotoxicity in SH-SY5Y neuroblastoma cells. Under normal serum condition, KMUP-1 enhanced protein expression of nNOS, PKG and sGCα1, increased intracellular cyclic GMP level, and attenuated PDE5 expression. KMUP-1 also increased expression of BDNF and Bcl-2, but it did not affect Bax expression. The phosphorylation of Akt and CREB induced by KMUP-1 was inhibited by tyrosine kinase (TrK) inhibitor K252a and phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, respectively. Under serum deprivation condition, flow cytometric analysis using Annexin V showed KMUP-1 increased cell viability, but lacked protective effects in the presence of nitric oxide synthase inhibitor L-NAME, PKG inhibitor Rp-8-pCPT-cGMPS or LY294002. KMUP-1 not only enhanced expression of nNOS, sGCα1, PKG, p-CREB, p-Akt and Bcl-2, but also attenuated Bax expression in serum deprivation-treated cultures. In conclusion, cGMP/PKG, PI3K/Akt/CREB and Bcl-2/Bax signals play critical roles in the neuroprotective effects of KMUP-1 on serum deprivation-induced toxicity.

1. Introduction

Recently, the potential role of cyclic guanosine monophosphate (cGMP) signaling pathway in preventing destruction and cell death in brain and peripheral nerve systems provide new direction for development of therapeutic agents for neurodegenerative disease, such as Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease (Fiscus, 2002; Makhinson et al., 2006; Takuma et al., 2001; Wang et al., 2005). cGMP-dependent protein kinase (PKG) is the major downstream target of cGMP and cGMP/PKG signaling pathway has been found to activate neural survival signal (Fiscus, 2002; Kim et al., 1999). Soluble guanylyl cyclase (sGC) is considered to be the major target of nitric oxide (NO) synthesized by neuronal nitric oxide synthases (nNOS) and a signal transduction enzyme that forms the second messenger molecular cGMP (McDonald and Murad, 1995).

KMUP-1 (7-[2-[4-(2-chlorophenyl) piperazinyl]ethyl]-1,3-dimethylxanthine), a chemical synthetic xanthine-based derivative, has been found to stimulate sGC/cGMP/PKG pathway and to inhibit phosphodiesterase (PDE)5 (Wu et al., 2001, 2004). PDE5 inhibitor plays a role in potentiating neurogenesis through the increase of cGMP levels (Bednar, 2008) and activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/GSK-3 pathway (Ghofrani et al., 2006). Serum deprivation- induced cell death is accompanied by down-regulation of important survival factors such as cGMP/PKG, PI3K/Akt systems and CREB phosphorylation (Ciani et al., 2002). Induction of cell death by growth factor or survival factor deprivation is an important process during neuronal development and has been used as a popular in vivo model for cell death studies (Pandey et al., 2003). Therefore, in this study, KMUP-1, a cGMP/PKG signal enhancer, was evaluated its protective effects on serum deprivation-induced neurotoxicity.

PI3K/Akt pathway is known to be important for neuronal survival and maintenance of many neuronal function such as long-term potentiation and memory formation (Frebel and Wiese, 2006; Karpova et al., 2006). The anti-apoptotic effects of PI3K are mediated by its downstream target Akt, which can regulate the expression of several apoptosis-related genes, such as

Abbreviations: BDNF, brain-derived neurotrophic factor; cGMP, cyclic guanosine monophosphate; CREB, cAMP response element-binding protein; nNOS, neuronal nitric oxide synthase; PDE, phosphodiesterase; PI3K, phosphatidylinositol 3-kinase; PKG, cGMP-dependent protein kinase; sGC, soluble guanylyl cyclase; Trk, tyrosine kinase.

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Bcl-2/Bax (Zheng et al., 2008). Up-regulation of Bcl-2 expression has been identified as a critical mechanism for cell survival (Pugazhenthi et al., 1999; Singleton et al., 1996; Tamatani et al., 1998). NO/cGMP/PKG signaling can prevent apoptosis via activation of the PI3K/Akt pathway (Ha et al., 2003; Haj et al., 2003) or via stimulation of the transcription factor CREB (Ciani et al., 2002). Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, plays an important role in promoting neuronal survival, neuronal differentiation, and synaptic plasticity (Lindsay et al., 1994; Thoenen, 1995). The action of BDNF is mediated by binding to the TrkB receptor (Biffo et al., 1995). TrkB, a protein tyrosine kinase receptor, was required for normal development and survival of neurons (Klein, 1994). Activation of TrkB receptor causes increased autophosphorylation and regulation of the PI3K (Yuan and Yankner, 2000). Directly modulating the CREB/BDNF/Bcl-2 cascade may have utility for the treatment of neuropsychiatric disorders (Nestler et al., 2002).

KMUP-1 possesses cGMP-dependent anti-proinflammatory (TNF-α) effects (Wu et al., 2006). Moreover, KMUP-1 enhances cGMP/PKG signaling, inhibits phosphodiesterases (PDEs), and activates K^+ channels resulting in relaxations in aortic (Wu et al., 2001), corporeal carvenosa (Lin et al., 2002), basilar artery myocytes (Wu et al., 2005) and prostate (Liu et al., 2007). In this study, we demonstrate that KMUP-1 activates cGMP/PKG, PI3K/Akt, Bcl-2/Bax and BDNF signal pathways and attenuates serum deprivation-induced neurotoxicity in SH-SY5Y cells.

2. Materials and methods

2.1. Materials

Human neuroblastoma cell line SH-SY5Y was purchased from ATCC (CRL-2266). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), brain-derived neurotrophic factor (BDNF), K252a, LY294002 and Rp-8-pCPT-cGMPS were purchased from Sigma–Aldrich (St. Louis, MO, USA). N^W-nitro-L-arginine methyl ester (L-NAME) was purchased from Research Biochemical International (Natick, MA, USA). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 Medium, fetal bovine serum (FBS), penicillin, amphotericin B and streptomycin were purchased from Invitrogen (Grand Island, NY, USA). KMUP-1, BDNF, Rp-8-pCPT-cGMPS and L-NAME were dissolved in distilled water. K252a and LY294002 were dissolved in DMSO.

2.2. Cell cultures and serum deprivation

The SH-SY5Y cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and 95% air and cultured in a 1:1 mixture of DMEM and Ham's F12 medium containing 10% (v/v) heat-inactivated FBS, 4 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL amphotericin B. For serum deprivation, cells were washed twice with PBS then incubated in serum-free medium containing 4 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL amphotericin B. Duration of each drug treatment is described in detail in the figure legends. Trk inhibitor K252a (200 nM), PI3K inhibitor LY294002 (30 μM), PKG inhibitor Rp-8-pCPT-cGMPS (10 μM) or L-NAME (10 μM) were respectively added for 30 min before cells were treated with KMUP-1.

2.3. Cell viability

Cell viability was measured by a quantitative colorimetric assay with MTT, which shows the mitochondrial activity of living cells. After treatment of KMUP-1 for 24 h, cells were incubated with 50 μL MTT (final concentration 0.5 mg/mL) for 3 h at 37 °C. The reaction was terminated by addition of 200 μL DMSO. The amount of MTT formazon product was determined by measuring the absorbance at 560 nm using a microplate reader (Molecular devices, Palo Alto, CA).

2.4. Western blotting analysis

KMUP-1, BDNF and various inhibitors were added as indicated in the figure legends. After treatment indicated, cells were collected and lysed for protein expression. The protein concentration was determined using the Bio-Rad protein assay kit. Equal amounts of protein were separated by a polyacrylamide gel (10%) and transferred to polyvinylidene difluoride membranes from Perkin Elmer (Boston, MA, USA). Nonspecific binding was blocked with Tris–buffered saline Tween-20 (TBS-T: 50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6) containing 5% nonfat milk for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with one of the following specific primary antibodies: PKG (1:500 dilution; Abcam, Cambridge, UK), BDNF (1:1000 dilution; Abcam, Cambridge, UK,), PDE5 (1:1000 dilution; BD Biosciences, San Jose, CA, USA), nNOS (1:1000 dilution; Santa Cruz, CA, USA), sGCα1 (1:500 dilution; Santa Cruz, CA, USA), sGCβ1 (1:1000 dilution; Santa Cruz, CA, USA), Akt (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA), CREB (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA), Bcl-2 (1:500 dilution; Chemicon, Temecula, CA, USA) and Bax (1:500 dilution; Chemicon, Temecula, CA, USA), phospho-Akt (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA), phospho-CREB (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA), GAPDH (1:20,000 dilution; Santa Cruz, CA, USA). Membranes were washed six times per 5 min with TBS-T. The appropriate dilutions of secondary antibodies (diluted 1:1000) were incubated for 1 h. Following six washes with TBS-T, the protein bands were detected with the ECL reagent from Perkin Elmer (Boston, MA, USA).

2.5. Determination of cGMP

SH-SY5Y cells were incubated with KMUP-1 (0.01, 0.05 and 0.1 μM) for 24 h. After incubation, the cells were extracted with 1 mL of 0.1N HCl. The extracts were then lyophilized for further measurement of cGMP in each sample using commercially available radioimmunoassay kits (Amersham Pharmacia Biotech, Buckinghamshire, England).

2.6. Apoptosis assays with Annexin V

Cells undergoing apoptosis were detected with the use of double staining with Annexin V-FITC/PI in dark according to the manufacturer's instructions. Briefly, cells attached to plastic dishes were harvested by 0.25% trypsin and washed twice with cold PBS. The cell pellets were suspended in 1× binding buffer (10 mM HEPES/NaOH, pH7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Then the cells were incubated with AnnexinV- FITC and propidium iodide (PI) for 15 min (22–25 °C) in dark. The stained cells were immediately analyzed by flow cytometry (Partec, Germany). Annexin V-FITC selectively passed through the plasma membranes of apoptotic cells and stained them with green fluorescence. Cells that were annexin V-negative and PI-negative were considered viable cells. Cells that were annexin V-positive and PI-negative were considered early apoptotic cells. Cells that were annexin V-positive and PI-positive were considered late apoptotic cells.

2.7. Statistical analysis

Data were expressed as mean ± S.E.M. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences followed by Dunnett's test for all pair's comparisons. A value of $p < 0.05$ was considered statistically significant. The

Table 1
Effects of nitric oxide synthase inhibitor L-NAME, PKG inhibitor Rp-8-pCPT-cGMPS and PI3K inhibitor LY294002 on KMUP-1-induced protection in serum deprivation-treated SH-SY5Y cells.

KMUP-1 (μM)	With serum	Without serum	Without serum		
			L-NAME	Rp-8-pCPT-cGMPS	LY294002
0	100% (CTL)	62.07 ± 5.08%#	–	–	–
0.01	99.32 ± 5.42%	82.76 ± 5.92%*	61.67 ± 5.88%ϕ	62.53 ± 5.88%ϕ	61.98 ± 5.73%ϕ
0.05	99.75 ± 5.65%	87.05 ± 5.43%*	60.87 ± 5.98%ϕ	63.23 ± 5.21%ϕ	61.72 ± 5.38%ϕ
0.1	100.97 ± 5.45%	91.65 ± 6.01%*	62.13 ± 5.13%ϕ	62.28 ± 5.38%ϕ	60.24 ± 5.12%ϕ
1	100.14 ± 5.51%	94.27 ± 5.42%*	61.67 ± 6.07%ϕ	64.02 ± 6.09%ϕ	61.46 ± 5.76%ϕ

Cells were treated with or without L-NAME (10 μM) or Rp-8-pCPT-cGMPS (10 μM) or LY294002 (30 μM) plus KMUP-1 in the absence of serum for 24 h. Cell viability was measured by MTT assay. Data are represented as percentages of the serum-containing cultures without any treatment (control group, CTL). Values represent the mean ± S.E.M. from six independent experiments. #$p < 0.05$ vs. CTL. *$p < 0.05$ vs. serum-deprivation cultures without any treatment. ϕ$p < 0.05$ vs. KMUP-1-treated serum deprivation groups. ANOVA followed by Dunnett's test.