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GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR–MEDIATED ENTERIC NEURONAL SURVIVAL INVOLVES GLYCOGEN SYNTHASE KINASE-3β PHOSPHORYLATION AND COUPLING WITH 14-3-3

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Abstract—Glial cell line-derived neurotrophic factor (GDNF) promotes the growth and survival of enteric neurons, but the mechanisms involved are poorly understood. GDNF is known to promote the survival of enteric neurons through activation of the PI3-Kinase/Akt signaling pathway. We investigated the role of glycogen synthase kinase-3β (GSK-3β) in enteric neuronal survival, and the ability of GDNF to regulate the activity of GSK-3β using primary rat embryonic enteric neurons. GDNF, through activation of the PI3-kinase pathway enhanced the phosphorylation of GSK-3β at its N-terminal serine-9 residue, and promoted the association of GSK-3β with 14-3-3. Transfection of a constitutively active S9A-GSK-3β mutant prevented the survival effects of GDNF, whereas a dominant negative GSK-3β construct prevented GDNF withdrawal-induced cell death. Increased GSK-3β activity was associated with an increase in tau phosphorylation. Thus, GDNF promotes enteric neuronal survival by modulating GSK-3β and its downstream target tau. Inhibitors of GSK-3β activity may have therapeutic potential in improving enteric neuronal survival.

Key words: GSK-3β, GDNF, 14-3-3, enteric, neurons, survival.

The enteric nervous system (ENS) is the largest and most complex division of the autonomic nervous system, and is involved in the control of gut movements essential for food propulsion as well as in the regulation of absorption and secretion by cells of the gut mucosa (Newgreen and Young, 2002a). The ENS consists of neurons and glial cells arranged into a network of regularly spaced ganglia connected by internodal strands within the walls of the gastrointestinal tract, and develops from vagal and sacral neural crest cells that migrate and colonize the entire gastrointestinal tract during embryonic development (Newgreen and Young, 2002a,b). The normal development of the ENS is controlled by signals from several signaling systems, particularly those from members of the glial cell line-derived neurotrophic factor (GDNF)/Ret/GDNF receptor family α (GFRα) signaling system (Chalazonitis et al., 1998; Hearn et al., 1998; Heuckeroth et al., 1998; Pachnis et al., 1998; Young et al., 2001). Defects in these systems have been shown to result in defects in ENS development similar to Hirschsprung's disease in which there is loss of enteric neurons in sections of the rectum, colon and sometimes the proximal ileum (Enomoto et al., 1998; Gariepy, 2001; Gianino et al., 2003; Natarajan et al., 2002).

GDNF is a member of a family of neurotrophic factors distantly related to TGF-β that support proliferation, survival and differentiation of neuronal precursor cells (Airaksinen and Saarma, 2002). GDNF was originally described as a trophic factor for embryonic mid-brain dopamine neurons, but is now known to be essential for the development and survival of other neuronal sub-populations including enteric, parasympathetic, motor and somatic sensory neurons (Heuckeroth et al., 1998; Natarajan et al., 2002). Although GDNF signaling through the PI3-kinase (PI3-K)/Akt signaling pathway is recognized as being critical for the survival of enteric neurons, the downstream targets involved are not well understood (Besset et al., 2000; Mograbi et al., 2001; Soler et al., 1999). We showed in a previous study that GDNF promotes enteric neuron survival and neurite extension through activation of the PI3-K signaling pathway (Srinivasan et al., 2005). We demonstrated that GDNF promotes survival of enteric neurons by stimulating the phosphorylation of Akt, which in turn phosphorylates the pro-apoptotic FOXO1 and FOXO3a transcription factors leading to their exclusion from the nucleus.

Glycogen synthase kinase-3β (GSK-3β) is a serine-threonine kinase that is involved in the signaling pathways of insulin and neurotrophic factors, and plays an important role in cell cycle regulation and proliferation. GSK-3β is constitutively active in resting cells and acts mainly by phosphorylating its substrates, and is inactivated by extracellular signals (Doble and Woodgett, 2003; Grimes and Jope, 2001; Jope and Johnson, 2004). Previous studies have shown that GSK-3β function is required for sympathetic and cortical neuron cell death due to inhibition of PI3-K, Akt, or trophic factor withdrawal (Crowder and Freeman, 2000; Hetman et al., 2000). However, the role of GSK-3β in GDNF-mediated enteric neuronal cell growth and survival is not known.

In this study we examined whether GDNF-mediated enteric neuronal survival involves suppression of GSK-3β

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Abbreviations: DN-GSK-3β, dead dominant negative glycogen synthase kinase-3β mutant; ENS, enteric nervous system; EV, empty PCS2+ vector; GDNF, glial cell line-derived neurotrophic factor; GSK-3β, glycogen synthase kinase-3β; PI3-K, PI3-kinase; WT-GSK-3β, wild-type glycogen synthase kinase-3β.

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activity. We demonstrate for the first time that GDNF supports enteric neuronal cell survival through its ability to promote the phosphorylation and inactivation of GSK-3β. Further we find that 14-3-3 proteins play a role in GDNF-mediated GSK-3β inactivation. We also show that GSK-3β promotes enteric neuronal cell death through a mechanism involving caspase-3 activation and tau hyperphosphorylation.

EXPERIMENTAL PROCEDURES

Materials

Recombinant rat GDNF was produced and purified as previously described (Creedon et al., 1997). Goat anti-mouse IgG microbeads and Macs (type MS) magnetic separation columns were purchased from Miltenyi Biotech (Auburn, CA, USA). LY294002 and cell lysis buffer were obtained from Cell Signaling Technology (Danvers, MA, USA). The 14-3-3 antagonist R18 peptide has previously been described (Wang et al., 1999).

Antibodies

Mouse monoclonal antibodies to β-actin (A5441, clone AC-15) were from Sigma-Aldrich (St. Louis, MO, USA), rat low affinity nerve growth factor receptor (p75NGFR) (MAB365, clone 192-IgG), GSK-3β (MAB8689) and peripherin from Chemicon International (Temecula, CA, USA), rabbit polyclonal antibodies to Akt, phospho-Akt (ser473), cleaved caspase-3 (Asp175), and phospho-GSK-3β (Ser9) from Cell Signaling Technology, and rabbit polyclonal antibodies to 14-3-3 β (FL-246) and phospho-Tau (Ser404) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase–conjugated goat anti-rabbit and anti-mouse IgG antibodies were from Cell Signaling Technology.

Primary culture

Primary cultures of enteric neurons were prepared from the stomach, small and large intestines of embryonic day 14.5 Sprague–Dawley rat embryos and maintained in N2 medium supplemented with or without 100 ng/ml GDNF as previously described (Heuckeroth et al., 1998; Srinivasan et al., 2005). After 24 h of culture of neurons isolated by this method, 80% of the cells stain positive for Ret indicating a neuronal phenotype (Srinivasan et al., 2005).

For GSK-3β phosphorylation studies, isolated enteric neurons were suspended in N2 medium supplemented with GDNF and plated at 3×10^5 cells/well in poly-D-lysine/laminin-coated six-well plates (BD Biosciences Discovery Labware, Bedford, MA, USA). The cells were incubated overnight at 37 °C in a humidified CO_2 incubator, washed three times with DMEM/F-12 medium (Gibco BRL, Grand Island, NY, USA), and incubated for a further 24 h in DMEM/F-12 medium without GDNF. The cells were incubated for a further 1 h at 37 °C in Krebs buffer (vehicle only and GDNF only treatments) or Krebs buffer containing LY294002 (50 μM) before stimulation for 30 min at 37 °C with GDNF (100 ng/mL) or GDNF plus LY294002 (50 μM) in fresh Krebs buffer.

For transfection and survival studies, isolated enteric neurons were seeded at 1.25×10^4 cells/well of an eight-well poly-D-lysine/laminin-coated culture slide (BD Labware) and 3×10^5 cells/well of a six well poly-D-lysine/laminin-coated plate in N2 medium supplemented with or without GDNF. The cells were allowed to stabilize before transfection by overnight incubation at 37 °C in a humidified CO_2 incubator.

Transfection of enteric neurons

Enteric neurons were transfected with *Xenopus* GSK-3β constructs (kind gifts from Dr. Peter Klein, University of Pennsylvania School of Medicine, Philadelphia, PA, USA), pEGFP vector (BD Biosciences Clontech, Mountain View, CA, USA) or control empty PCS2+ vector (EV) using Lipofectamine 2000 (Invitrogen Lifetechnologies, Carlsbad, CA, USA) according to the manufacturer's recommended procedure. The GSK-3β constructs were created in the PCS2 (+) vector and included wild-type glycogen synthase kinase-3β (WT-GSK-3β), an S9A GSK-3β mutant (S9A-GSK-3β) in which the N-terminal serine nine residue had been substituted with an alanine residue, and a kinase dead dominant negative glycogen synthase kinase-3β mutant (DN-GSK-3β) in which a conserved lysine residue (K85) in the ATP-binding region had been substituted with an arginine residue (Pierce and Kimelman, 1995). To monitor transfection efficiencies the constructs were co-transfected with pEGFP vector at a 1:1 ratio. Cells cultured in eight-well culture slides were transfected with 0.05 μg vector DNA and 0.2 μl Lipofectamine 2000/well, while those cultured in six-well plates were transfected with 1.5 μg vector DNA and 5 μl Lipofectamine 2000/well. The cells were cultured for 24 h at 37 °C in N2 medium with or without GDNF after transfection, and either lysed for Western blotting or fixed for immunocytochemistry.

Western blotting

Enteric neurons were lysed with 1× cell lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany), PMSF (1 mM, Fluka, Buchs, Switzerland) and okadaic acid (50 mM, Roche Applied Science), and the lysates stored at −80 °C until used. Western blotting was performed according to standard procedure. Briefly, protein concentrations were determined according to the Lowry method using the D_C protein assay kit reagents (Bio-Rad Laboratories, Hercules, CA, USA), and between 5 and 20 μg of protein was separated electrophoretically on 10–20% tris–glycine SDS-PAGE gradient gels (Bio-Rad). Resolved proteins were transferred to PVDF membranes (Bio-Rad), the membranes probed with the appropriate antibodies, and the proteins visualized by chemiluminescence with autoradiography using the ECL Plus Western Blotting Detection kit reagents (Amersham Biosciences, Piscataway, NJ, USA). The films were scanned and band densities analyzed using the Scion Image for Windows software (Scion Corporation, Frederick, MD, USA).

Co-immunoprecipitation of 14-3-3

14-3-3 proteins were immunoprecipitated from lysates of enteric neurons treated for 30 min with vehicle, GDNF or GDNF+ LY294002 (50 μM) using a mouse anti-GSK-3β monoclonal antibody (1 μg MAB8689/60 μg total protein) and protein G Plus agarose (Santa Cruz). The co-precipitation of 14-3-3 was analyzed by Western blotting using rabbit anti-14-3-3β polyclonal antibody. To determine the specificity of the interaction between phospho-GSK-3β and 14-3-3, lysates from GDNF-stimulated and non-stimulated cells were pre-incubated for 2 h at 4 °C with R18 peptide (20 μM) (Wang et al., 1999) before immunoprecipitation with the anti-GSK-3β monoclonal antibody.

Immunocytochemistry

Immunofluorescence microscopy was performed as previously described (Srinivasan et al., 2005). Briefly, enteric neurons were cultured in eight-well culture slides and transfected as described above. Neurons were fixed with 4% paraformaldehyde in PBS, permeabilized with Triton X-100 (0.1%) in PBS–citrate buffer (0.1%), and blocked for 1 h at room temperature with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 1% BSA (Sigma-Aldrich) in TBS–Triton-X (0.2% Triton-X). The slides were incubated overnight at 4 °C with primary antibody (1:50 dilution for cleaved caspase-3 antibody and anti-phospho-tau antibody) in TBS-T (containing 5% BSA and