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Activation of transglutaminase 2 by nerve growth factor in differentiating neuroblastoma cells: A role in cell survival and neurite outgrowth

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ABSTRACT

NGF (nerve growth factor) and tissue transglutaminase (TG2) play important roles in neurite outgrowth and modulation of neuronal cell survival. In this study, we investigated the regulation of TG2 transamidase activity by NGF in retinoic acid-induced differentiating mouse N2a and human SH-SY5Y neuroblastoma cells. TG2 transamidase activity was determined using an amine incorporation and a peptide cross linking assay. In situ TG2 activity was assessed by visualising the incorporation of biotin-X-cadaverine using confocal microscopy. The role of TG2 in NGF-induced cytoprotection and neurite outgrowth was investigated by monitoring hypoxia-induced cell death and appearance of axonal-like processes, respectively. The amine incorporation and protein crosslinking activity of TG2 increased in a time and concentration-dependent manner following stimulation with NGF in N2a and SH-SY5Y cells. NGF mediated increases in TG2 activity were abolished by the TG2 inhibitors Z-DON (Z-ZON-Val-Pro-Leu-OMe; Benzyloxycarbonyl-(6-Diazo-5-oxonorleucinyl)-L-valinyl-L-prolinyl-L-leucinmethylester) and R283 (1,3,dimethyl-2[2-oxo-propyl]thio)imidazole chloride) and by pharmacological inhibition of extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase B (PKB) and protein kinase C (PKC), and removal of extracellular Ca^{2+}. Fluorescence microscopy demonstrated NGF induced in situ TG2 activity. TG2 inhibition blocked NGF-induced attenuation of hypoxia-induced cell death and neurite outgrowth in both cell lines. Together, these results demonstrate that NGF stimulates TG2 transamidase activity via a ERK1/2, PKB and PKC-dependent pathway in differentiating mouse N2a and human SH-SY5Y neuroblastoma cells. Furthermore, NGF-induced cytoprotection and neurite outgrowth are dependent upon TG2. These results suggest a novel and important role of TG2 in the cellular functions of NGF.

1. Introduction

Transglutaminases (TGs) are a family of Ca^{2+}-dependent enzymes that catalyse the post-translational modification of proteins (Nurminskaya and Belkin, 2012; Eckert et al., 2014). There are eight distinct catalytically active members of the TG family which exhibit differential expression (Factor XIIIa and TGs 1–7).

The ubiquitously expressed TG2, which is the most widely studied member of the TG family, is involved in the regulation of numerous cellular processes, including cell adhesion, migration, growth, survival, apoptosis, differentiation, and extracellular matrix organization (Nurminskaya and Belkin, 2012; Eckert et al., 2014). In neuronal cells, TG2 is involved in neurite outgrowth during differentiation and in neuroprotection following cerebral ischaemia (Tucholski et al., 2001; Filiano et al., 2010; Vanella et al., 2015).

Transglutaminase 2 possesses multiple enzymic functions that include transamidation, protein disulphide isomerase and protein kinase activity (Gundemir et al., 2012). Furthermore, TG2 also has non-enzymatic functions which can modulate signal transduction pathways (Nurminskaya and Belkin, 2012).

Receptor tyrosine kinases represent a large family of receptors whose prominent members include receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). It is notable that cytoplasmic TG2-mediated transamidase activity participates in EGF receptor signalling, whereas the interaction of extracellular TG2 with PDGF and VEGF receptors promotes their activation (Dardik and Inbal, 2006; Zemskov et al., 2009; Li et al., 2010). These observations suggest a major role for TG2 in the modulation of receptor tyrosine kinases. However, at present, it is not known if receptor tyrosine kinase activation promotes intracellular TG2 activation. A study has shown that prolonged exposure (3–6 days) of mouse N2a neuroblastoma cells to nerve growth factor (NGF) promoted increased TG2 protein expression and TG2-mediated transamidase activity (Condello et al., 2008). However, it is conceivable that the increased levels of transamidase activity may reflect increased levels of TG2 expression rather than direct activation of

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the enzyme itself by NGF-induced signalling. NGF triggers its biological effects via the tyrosine kinase receptor TrkA (Wang et al., 2014), which when activated stimulates a multitude of signalling pathways including ERK1/2 (extracellular signal-regulated kinases 1 and 2), PI-3K (phosphatidylinositol 3-kinase)/PKB (protein kinase B) and PLC-γ (phospholipase C-γ)/PKC (protein kinase C) cascades (Wang et al., 2014). As some of these pathways are associated with modulation of intracellular TG2 activity (PKC, ERK1/2 and Ca^{2+}) it is conceivable that NGF directly regulates TG2 activity. Since mouse N2a and human SH-SY5Y neuroblastoma cells are responsive to NGF (Price et al., 2003; Condello et al., 2008; Dwane et al., 2013), the primary aims of this study were (i) to determine whether short term treatment with NGF (< 4 h) could modulate TG2-mediated transamidase activity in these cells and (ii) to assess the role of TG2 in NGF-induced neuroprotection and neurite outgrowth. The results obtained indicate that NGF triggers robust TG2-mediated amine incorporation and protein cross-linking activity in mouse N2a and human SH-SY5Y cells. Furthermore, inhibition of TG2 attenuated NGF-induced cytoprotection and neurite outgrowth. Overall, these results suggest a novel and prominent role for TG2 in NGF function and signalling.

2. Materials and methods

2.1. Materials

Nerve growth factor (NGF) was obtained from Merck Millipore (Watford, UK). Akt inhibitor XI was purchased from Calbiochem (San Diego, CA). BAPTA/AM (1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis acetoxymethyl ester), PD 98059 (2′-amino-3′-methoxyflavone) and Ro 31-8220 (3-[3-[2,5-Dihydro-4-(1-methyl-1*H*-indol-3-yl)-2,5-dioxo-1*H*-pyrrol-3-yl]-1*H*-indol-1-yl]propyl carbamimidothioic acid ester mesylate) were obtained from Tocris Bioscience (Bristol, UK). *All-trans* retinoic acid, casein, Protease Inhibitor Cocktail (for use with mammalian cell and tissue extracts), Phosphatase Inhibitor Cocktail 2 and 3, hordereadish peroxidase conjugated-ExtrAvidin® (ExtrAvidin®-HRP) and fluorescein isothiocyanate conjugated ExtrAvidin® (ExtrAvidin®-FITC) were obtained from Sigma-Aldrich Co. Ltd. (Gillingham, UK). The TG2 inhibitors Z-DON (Z-ZON-Val-Pro-Leu-OMe; Benzyloxycarbonyl-(6-Diazo-5-oxonorleucinyl)-L-valinyl-L-prolinyl-L-leucinmethylester) and R283 (1,3,dimethyl-2[2-oxopropyl]thio)imidazole chloride), together with purified guinea-pig liver TG2 were obtained from Zedira GmbH (Darmstadt, Germany). DAPI (4′,6-diamidino-2-phenylindole) was from Vector Laboratories Inc (Peterborough, UK). Biotin-TVQQEL was purchased from Pepceuticals (Enderby, UK). Biotin cadaverine (N-(5-aminopentyl)biotinamide) and biotin-X-cadaverine (5-([[N-(biotinoyl)amino)hexanoyl]amino)pentylamine) were purchased from Invitrogen (Loughborough, UK). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum, trypsin (10×), L-glutamine (200 mM), penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) were purchased from Scientific Laboratory Supplies (Nottingham, UK. All other reagents were purchased from Sigma-Aldrich Co. Ltd. (Gillingham, UK) and were of analytical grade.

Antibodies were obtained from the following suppliers: monoclonal anti-phospho ERK1/2 (Thr^{202}/Tyr^{204}) from Sigma-Aldrich Co. Ltd. (Gillingham, UK); polyclonal anti-phospho PKB (Ser^{473}), polyclonal anti-total PKB, monoclonal anti-total ERK1/2, and polyclonal anti-cleaved caspase 3 from New England Biolabs Ltd. (Hitchin, UK); monoclonal anti-TG2 (CUB 7402) from Thermo Scientific (Loughborough, UK); polyclonal anti-human keratinocyte TG1 and polyclonal anti-human epidermal TG3 from Zedira GmbH (Darmstadt, Germany); monoclonal anti-GAPDH from Santa Cruz Biotechnology Inc (Heidelberg, Germany); Alexa Fluor® 488 goat anti-mouse IgG labelled secondary antibody from Thermo Scientific (Loughborough, UK).

2.2. Cell culture

Murine N2a and human SH-SY5Y neuroblastoma cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). Cells were cultured in DMEM supplemented with 2 mM L-glutamine, 10% (v/v) foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were maintained in a humidified incubator (95% air/5% CO_2 at 37 °C) until 70–80% confluent and sub-cultured (1:5 split ratio) every 3–4 days. SH-SY5Y cells were sub-cultured using trypsin (0.05% w/v)/EDTA (0.02% w/v). Differentiation of N2a cells was induced by culturing cells in serum-free DMEM containing 1 μM *all-trans* retinoic acid for 48 h, unless otherwise specified. Differentiation of SH-SY5Y cells was induced by culturing cells in serum-free DMEM containing 10 μM *all-trans* retinoic acid for 5 days. Experiments were performed on passage numbers 8–20 for N2a and 18–25 for SH-SY5Y.

2.3. Cell extraction for measurement of TG2 activity

Following prior differentiation with retinoic acid as described above time course profiles and concentration-response curves were obtained for NGF. Where appropriate, cells were also pre-incubated for 30 min in medium with or without the protein kinase inhibitors Akt inhibitor XI (PKB/Akt, 100 nM; Barve et al., 2006), PD 98059 (MEK1/2, 50 μM; Dudley et al., 1995), and Ro 31-8220 (PKC, 10 μM; Davis et al., 1989) prior to treatment with 100 ng/ml NGF. The concentrations of protein kinase inhibitors employed in this study were in the range of values in the literature that are used to inhibit the cellular activity of these kinases: PD 98059 (10–50 μM; Sutter et al., 2004; Kim et al., 2008), Akt inhibitor XI (1 μM; Frampton et al., 2012; Rybchyn et al., 2011) and Ro 31-8220 (1–10 μM; Lee et al., 2013; Montejo-López et al., 2016). In the case of less well known Akt inhibitor XI, effects on PKB inhibition were verified by Western blot analysis.

Following stimulation with NGF, N2a and SH-SY5Y cells were rinsed twice with 2.0 ml of chilled PBS, lysed with 500 μl of ice-cold lysis buffer ((50 mM Tris-HCl pH 8.0, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) Protease Inhibitor Cocktail, and 1% (v/v) Phosphatase Inhibitor Cocktail 2)). Cell lysates were clarified by centrifugation at 4 °C for 10 min at 14,000 × *g* prior to being assayed for TG transamidase activity. Supernatants were collected and stored at − 80 °C.

Protein levels were determined by the bicinchoninic acid (BCA) protein assay, based on the method of Smith et al. (1985), which was performed using a commercially available kit (Sigma-Aldrich Co. Ltd, UK) using bovine serum albumin (BSA) as the standard. Transglutaminase activity was subsequently monitored by two different transamidase assays; amine incorporation and protein cross-linking.

2.4. Biotin-labelled cadaverine incorporation assay

The assay was performed as per the method described by Slaughter et al. (1992) with the modifications of Lilley et al. (1998). Briefly, 96-well microtitre plates were coated overnight at 4 °C with 250 μl of N′,N′-dimethylcasein (10 mg/ml in 100 mM Tris-HCl, pH 8.0). The plate was washed twice with distilled water and blocked with 250 μl of 3% (w/v) BSA in 100 mM Tris-HCl, pH 8.0 and incubated for 1 h at room temperature. The plate was washed twice before the application of 150 μl of either 6.67 mM calcium chloride and or 13.3 mM EDTA (used to deplete calcium and suppress TG activity) assay buffer containing 225 μM biotin cadaverine (a widely used substrate to monitor TG amine incorporating activity) and 2 mM 2-mercaptoethanol. The reaction was started by the addition of 50 μl of samples or positive