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A_1 adenosine receptor-induced phosphorylation and modulation of transglutaminase 2 activity in H9c2 cells: A role in cell survival

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ABSTRACT

The regulation of tissue transglutaminase (TG2) activity by the GPCR family is poorly understood. In this study, we investigated the modulation of TG2 activity by the A_1 adenosine receptor in cardiomyocyte-like H9c2 cells. H9c2 cells were lysed following stimulation with the A_1 adenosine receptor agonist N^6-cyclopentyladenosine (CPA). Transglutaminase activity was determined using an amine incorporating and a protein cross linking assay. TG2 phosphorylation was assessed via immunoprecipitation and Western blotting. The role of TG2 in A_1 adenosine receptor-induced cytoprotection was investigated by monitoring hypoxia-induced cell death. CPA induced time and concentration-dependent increases in amine incorporating and protein crosslinking activity of TG2. CPA-induced increases in TG2 activity were attenuated by the TG2 inhibitors Z-DON and R283. Responses to CPA were blocked by PKC (Ro 31-8220), MEK1/2 (PD 98059), p38 MAPK (SB 203580) and JNK1/2 (SP 600125) inhibitors and by removal of extracellular Ca^{2+}. CPA triggered robust increases in the levels of TG2-associated phosphoserine and phosphothreonine, which were attenuated by PKC, MEK1/2 and JNK1/2 inhibitors. Fluorescence microscopy revealed TG2-mediated biotin-X-cadaverine incorporation into proteins and proteomic analysis identified known (Histone H4) and novel (Hexokinase 1) protein substrates for TG2. CPA pre-treatment reversed hypoxia-induced LDH release and decreases in MTT reduction. TG2 inhibitors R283 and Z-DON attenuated A_1 adenosine receptor-induced cytoprotection. TG2 activity was stimulated by the A_1 adenosine receptor in H9c2 cells via a multi protein kinase dependent pathway. These results suggest a role for TG2 in A_1 adenosine receptor-induced cytoprotection.

1. Introduction

Transglutaminases (TGs) comprise a family of calcium (Ca^{2+}) dependent enzymes (TG1-7 and Factor XIIIa) that catalyse post-translational modification of proteins. Once Ca^{2+} binds to TG, a cysteine is exposed leading to the formation of a bond between ε-amide (as an isodipeptide or polyamine bond) and γ-carboxamide of protein bound glutamine residues [1]. Transglutaminase 2 (TG2), the most widely studied member of the TG family, has been implicated in the regulation of a wide range of processes, including cell adhesion, migration, growth, survival, apoptosis, differentiation, and extracellular matrix organisation [2]. The role of TG2 in cell survival and cell death is cell-specific with respect to whether it has pro- or anti-apoptotic effects [3]. Dysregulation of TG2 occurs in many pathologies, including coeliac disease, neurodegenerative disorders, some cancers and, as such, represents a potential therapeutic target [4].

Abbreviations: BSA, bovine serum albumin; CPA, N^6-cyclopentyladenosine; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EDTA, ethylenediaminetetraacetic acid; ERK1/2, extracellular signal-regulated kinases 1 and 2; FITC, fluorescein isothiocyanate; GPCRs, G-protein coupled receptors; HRP, horseradish peroxidase; IGEPAL CA-630, octylphenyl-polyethylene glycol; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen activated protein kinase; MEK1/2, mitogen-activated protein kinase kinase 1/2; MKK4/7, mitogen activated protein kinase kinase 4 and 7; MTT, 3-(4-5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PD 98059, 2′-amino-3′-methoxyflavone; PI-3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; R283, 1,3-dimethyl-2[(2-oxopropyl)thio]imidazolium chloride; Ro 31-8220, 3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SP 600125, anthra[1-9-cd]pyrazol-6 (2H)-one; SWATH-MS, Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra; TG2, transglutaminase type 2; Z-DON, benzyloxycarbonyl-(6-diazo-5-oxonleucinyl)-L-valiyl-L-prolinyl-L-leucinmethylester.

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Transglutaminase 2 possesses multiple enzymic functions that include transamidation, protein disulphide isomerase and protein kinase activity [5]. The transamidase activity of TG2 is inhibited by GTP/GDP and when bound to GTP/GDP, TG2 functions as a G-protein known as Gh independently of its transamidase activity [6]. Interestingly, the activity of TG2 and other TGs can be regulated by protein kinases. For example, phosphorylation of TG2 by protein kinase A (PKA) inhibits its transamidating activity but enhances its kinase activity [7], whereas cross-linking activity of TG1 is enhanced by phorbol ester-induced stimulation of protein kinase C (PKC) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) [8]. Finally, PKC-δ has been shown to regulate TG2 expression in pancreatic cancer cells [9]. Overall, these observations suggest that the activity and expression of specific TG isoenzymes can be regulated by signalling pathways associated with G-protein coupled receptors (GPCRs). However, little is currently known about the regulation of TG2 enzymic activity following GPCR stimulation.

The A_1 adenosine receptor is a member of the GPCR superfamily, which couples to pertussis toxin-sensitive G_i/G_o-proteins [10]. Although the A_1 adenosine receptor stimulation is traditionally associated with inhibition of adenylyl cyclase, it also triggers the activation of additional signalling cascades involving PKC, PKB, ERK1/2, and p38 MAPK [11–16]. Since PKC and ERK1/2 pathways are associated with modulation of TG activity [7,8], it is conceivable that the A_1 adenosine receptor regulates TG activity. Since H9c2 cells express functional A_1 adenosine receptors [17] the primary aims of this study were (i) to determine whether the A_1 adenosine receptor modulates TG2 activity in these cells, and (ii) whether TG2 is involved in A_1 adenosine receptor induced cytoprotection [17]. The results obtained indicate that A_1 adenosine receptor stimulation modulates TG2 phosphorylation and activity via a multi protein kinase and extracellular Ca^{2+}-dependent pathway.

2. Materials and methods

2.1. Materials

BAPTA/AM, forskolin, PD 98059, Ro-31-8220 ({3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide bisindolylmaleimide IX, methanesulphonate}) SB 203580, SP 600 125, and thapsigargin were obtained from Tocris Bioscience (Bristol, UK). Adenosine, casein, DPCPX (1,3-dipropylcyclopentylxanthine), IGEPAL, MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), N^6-cyclopentyladenosine, N′,N′-dimethylcasein, paraformaldehyde, pertussis toxin, protease inhibitor cocktail, phosphatase inhibitor cocktail 2 and 3, ExtrAvidin®-HRP and ExtrAvidin®-FITC and Triton™ X-100 were obtained from Sigma–Aldrich Co. Ltd. (Gillingham, UK). Fluo-8/AM was purchased from Stratech Scientific Ltd (Newmarket, UK). The TG2 inhibitors Z-DON (Z-ZON-Val-Pro-Leu-OMe) and R283 along with purified standard guinea-pig liver TG2 were obtained from Zedira GmbH (Darmstadt, Germany). Biotin-TVQQEL was purchased from Pepceuticals (Enderby, UK). DAPI was from Vector Laboratories Inc (Peterborough, UK). Coomassie blue (InstantBlue™ stain) was purchased from Expedeon (Swavesey, UK). Biotin cadaverine (N-(5-Aminopentyl) biotinamide) and biotin-X-cadaverine(5-([(N-(Biotinoyl)amino)hexa noyl]amino)pentylamine) were purchased from Invitrogen UK (Loughborough, UK). DMEM (Dulbecco's modified Eagle's medium), foetal bovine serum, trypsin (10X), L-glutamine (200 mM), penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) were purchased from Lonza, (Castleford, 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 phospho-specific ERK1/2 (Thr^{202}/Tyr^{204}) from Sigma–Aldrich Co. Ltd; polyclonal phospho-specific PKB (Ser^{473}), polyclonal total unphosphorylated PKB, monoclonal total unphosphorylated ERK1/2, polyclonal total unphosphorylated JNK, polyclonal total unphosphorylated p38 MAPK, monoclonal phospho-specific p38 MAPK and monoclonal phospho-specific JNK were from New England Biolabs (UK) Ltd (Hitchin, UK); monoclonal anti-TG2 (CUB 7402) from Thermo Scientific (Leicestershire, UK); polyclonal anti-phosphoserine and polyclonal anti-phosphothreonine from Abcam (Cambridge, UK).

2.2. Cell culture

Rat embryonic cardiomyoblast-derived H9c2 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). These cells, derived from embryonic rat heart tissue [18], are increasingly used as an *in vitro* model for studies exploring cardioprotection since they display similar morphological, electrophysiological and biochemical properties to primary cardiac myocytes [19]. Cells were cultured in DMEM supplemented with 2 mM L-glutamine, 10% (v/v) foetal bovine serum and penicillin (100 U/ml)/streptomycin (100 μg/ml). They were maintained in a humidified incubator (95% air/5% CO_2 at 37 °C) until 70–80% confluence and sub-cultured (1:5 split ratio) using trypsin (0.05% w/v)/EDTA (0.02% w/v). Experiments were performed on passage numbers 6–23.

2.3. Transglutaminase activity assays

Time course profiles and concentration–response curves were obtained for CPA and adenosine. Where appropriate, cells were also pre-incubated for 30 min in a medium with or without the protein kinase inhibitors Ro 31-8220 (PKC; 10 μM; [20]), PD 98059 (MEK1/2, 50 μM; [21]), SB 203580 (p38 MAPK; 20 μM; [22]), and SP 600 125 (JNK1/2; 20 μM; [23]) prior to treatment with 100 nM CPA or 100 μM adenosine. Following stimulation, 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 TG2 activity, as described below. Protein was determined using the bicinchoninic acid (BCA) protein assay [24], using a commercially available kit (Sigma–Aldrich Co. Ltd), with bovine serum albumin (BSA) as the standard.

Biotin-labelled cadaverine-incorporation assays were performed according to Slaughter et al. [25] with modifications [26], as described previously [27]. The biotin-labelled peptide cross-linking assay was performed according to the method of Trigwell et al. [28] with minor modifications [27]. The reaction was started by the addition of 50 μl of samples, positive control (50 ng/well of guinea-pig liver TG2) or negative control (100 mM Tris–HCl, pH 8.0) and allowed to proceed for 1 h at 37 °C. In both assays, the reaction was terminated by adding 50 μl of 5.0 M sulphuric acid and the absorbance read at 450 nm. One unit of TG2 was defined as a change in absorbance of one unit h^{-1}.

2.4. Hypoxia-induced cell death

H9c2 cells in glucose-free and serum-free DMEM (Gibco™, Life Technologies Ltd, Paisley, UK) were exposed to 8 h hypoxia using a hypoxic incubator (5% CO_2/1% O_2 at 37 °C) in which O_2 was replaced by N_2.