Cellular Signalling 51 (2018) 59–71

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Inhibition of cytokine-mediated JNK signalling by purinergic $P2Y_{11}$ receptors, a novel protective mechanism in endothelial cells

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ARTICLE INFO

Keywords:
G-protein coupled receptors
Interleukin-1β
C-Jun N-terminal kinase
Purinergic receptors
Inflammation

Chemical compounds studied in this article:
YM-254890 (PubChem CID: 9919454)
H-89 (PubChem CID: 449241)
NF340 (PubChem CID: 73755007)
MRS2179 (PubChem CID: 24867852)
ZM241385 (PubChem CID: 176407)

ABSTRACT

Previous research from our laboratory has demonstrated a novel phenomenon whereby GPCRs play a role in inhibiting cytokine-mediated c-Jun N-terminal kinase (JNK) signalling. So far this novel phenomenon seems to have been vastly overlooked, with little research in the area. Therefore, in this study we explored this further; by assessing the potential of P2YRs to mediate inhibition of cytokine-mediated JNK signalling and related functional outcomes in human endothelial cells. We utilised primary endothelial cells, and employed the use of endogenous activators of P2YRs and well characterised pharmacological inhibitors, to assess signalling parameters mediated by P2YRs, Interleukin-1β (IL-1β), TNFα and JNK. Activation of P2YRs with adenosine triphosphate (ATP) resulted in a time- and concentration-dependent inhibition of IL-1β-mediated phosphorylation of JNK and associated kinase activity. The effect was specific for cytokine-mediated JNK signalling, as ATP was without effect on JNK induced by other non-specific activators (e.g. sorbitol, anisomycin), nor effective against other MAPK pathways such as p38 and the canonical NFκB cascade. Pharmacological studies demonstrated a role for the $P2Y_{11}$ receptor in mediating this effect, but not the $P2Y_1$ nor the adenosine receptors (A1, A2A, A2B & A3). The novel $G\alpha_{q/11}$ inhibitor YM254890 and a protein kinase A (PKA) inhibitor H89 both partially reversed ATP-mediated inhibition of IL-1β-stimulated JNK indicating involvement of both $G\alpha_{q/11}$ and $G\alpha_s$ mediated pathways. ATP also partially reversed IL-1β-mediated induction of cyclo-oxygenase-2 (COX-2) and E-selectin. Collectively, these studies indicate the potential for activation of purinergic receptors to protect the endothelium from inflammatory driven JNK activation and may be a new target for inflammatory disease therapy.

1. Introduction

Purinergic P2Y receptors are an important subclass of G-protein coupled receptors and comprises of eight mammalian subtypes ($P2Y_{1,2,4,6,11,12,13}$ and $_{14}$) which confer different binding affinities for ATP, ADP, UTP, and UDP [1]. For example, the preferred agonist for $P2Y_{1,12}$ and $_{13}$ is ADP, whereas ATP and UTP are equipotent for $P2Y_2$ and the $P2Y_{11}$ receptor is the only one selective for ATP [2]. Of the P2Y subtypes, $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ are predominantly expressed in epithelial cells and play a functional role in these systems [3]. ATP was first thought to be solely an intracellular energy source with concentrations of around 5–10 mM in the cytoplasm of all cells; however it soon became clear that these intracellular nucleotides can be released not only following tissue injury but also by non-lytic mechanisms through regulated transport [4].

Early studies recognised that ATP and adenosine were particularly important in the mechanisms underlying local control of vessel tone [5], as well as cell migration, differentiation and death during angiogenesis, atherosclerosis, and restenosis following angioplasty [6, 7]. Extracellular ATP has also been shown to drive systemic inflammation, and tissue damage in murine models of LPS-induced inflammation [8]. More recently purinergic signalling has been shown to play a role in inflammation and cancer, where activation of $P2Y_{11}$ inhibits the migration of tumour derived endothelial cells via cAMP signalling [9].

Abbreviations: A2A, adenosine-2-A receptor; ATP, adenosine tri-phosphate; JNK, c-Jun N-terminal kinase; COX-2, cyclo-oxygenase-2; E-selectin, endothelial-leukocyte adhesion molecule-1; GPCR, G-protein coupled receptor; HCAEC, human coronary artery endothelial cells; HUVEC, human umbilical vein endothelial cells; ICAM, intracellular adhesion molecule; IP_3, inositol triphosphate; MDA-MB-231, Human Caucasian breast adenocarcinoma; PAR-2, proteinase-activated receptor-2; PKA, protein kinase A; P2YR, purinergic receptor; TNFα, tumour necrosis factor-alpha

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https://doi.org/10.1016/j.cellsig.2018.07.016
Received 17 April 2018; Received in revised form 23 July 2018; Accepted 31 July 2018
Available online 02 August 2018
0898-6568/

The cellular actions of $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ have been shown to be mediated through the activation of a number of key signalling pathways. These include the extracellular regulated protein kinases, namely ERK [1] and the stress-activated protein kinases, p38 MAP kinase, and c-Jun N-terminal kinase (JNK) [10, 11], however, the exact mechanisms involved in P2YR activation of these pathways are still not fully understood. In Human umbilical vein endothelial cells (HUVECs), activation of $P2Y_1$ leads to both p38 MAPK and JNK phosphorylation [11]. Of the three subtypes described so far, $P2Y_{11}$ is the most abundantly expressed in HUVECs [3] and its activation leads to both IP_3 and cAMP accumulation, demonstrating its interaction with both $G\alpha_{q/11}$ and $G\alpha_S$ [12, 13].

In previous studies we uncovered a novel mechanism of regulation of cytokine-mediated inflammatory signalling at the level of c-Jun N-terminal kinase (JNK). Pre-activation of either PAR2 or the $P2Y_2$ receptor mediates inhibition of TNFα-mediated JNK signalling, thought to be mediated in part by the dissociation of the TNFR1 from receptor associated proteins [14–16]. However these studies were conducted in clonal cell lines expressing high levels of receptor and it is unclear if this applies to endogenous levels of receptor or has a functional consequence.

Therefore, in this study we examined the potential for endogenous P2Y receptor activation to mediate inhibition of cytokine-stimulated JNK signalling in cultures of primary endothelial cells.

2. Material and methods

2.1. Materials

All materials used were of the highest commercial grade available and were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. The $P2Y_{11}$ receptor antagonist, NF340 (pIC_{50} 6.43 and 7.14 in Ca^{2+} and cAMP respectively, [17]) and the $P2Y_1$ receptor antagonist, MRS2179 (K_B = 100 nM, [18]) were obtained from Tocris Bioscience (R &D Systems Europe, Ltd., Abingdon, UK). The ecto-nucleotidase inhibitor, ARL67156 (pIC_{50} = 4.62 in human blood, [19]), the A2A antagonist ZM241385 (pIC_{50} = 9.52, [20]), the protein kinase A (PKA) inhibitor, H89 (IC_{50} = 135 nM, [21]) and the EPAC activator, 8-CPT-2Me-cAMP (EC_{50} = 2.2, [22]) were all from Tocris bioscience (R&D Systems Europe, Ltd., Abingdon, UK). Antibodies raised against phosphorylated forms of JNK (Thr 183/Tyr 185) and p65 (Ser 536) were from New England Biolabs (England, UK) and p38 MAP kinase (pTpY 180/182) was purchased from Invitrogen (Paisley, UK). Anti-IκBα, anti-JNK, anti-p65 and p38 MAP kinase antibodies were obtained from Santa Cruz Biotechnology (CA, USA). The horse radish peroxidase HRP-coupled secondary antibodies were manufactured by Jackson Immuno research and distributed from Stratech Scientific (Suffolk, UK). Tumour necrosis factor-α (TNFα) and interleukin-1-β (IL-1β) were purchased from Insight Biotechnology (Middlesex, UK). The YM-254890 compound [23] was a kind gift of Astellas Pharma. Inc., Japan. The Human sE-selectin ELISA kit was purchased from Invitrogen (Paisley, UK).

2.2. Cell culture

HUVECs (Lonza, UK, *CC-2519) were grown in endothelial basal media, supplemented with endothelial growth media (EGM-2) containing single aliquots (2% foetal bovine serum, 0.2 ml hydrocortisone, 2 ml hFGF-B, 0.5 ml VEGF, 0.5 ml R3-insulin like growth factor-1, 0.5 ml ascorbic acid, 0.5 ml hEGF, 0.5 ml GA 1000, 0.5 ml heparin *CC-3162) and were purchased from Lonza, UK (Each aliquot of cryopreserved HUVECs consisted of 3 pooled donors, and 3 vials were used in total for duration of experiments, 9 donors). Human coronary artery endothelial cells (HCAECs) (Promocell, Heidelberg) were grown in endothelial cell growth medium MV2 supplemented with 5% foetal calf serum, 5 ng/ml hEGF, 10 ng/ml hBFGF, 20 ng/ml insulin-like growth factor, 0.5 ng/ml hVEGF, 1 μg/ml ascorbic acid and 0.2 μg/ml hydrocortisone (each aliquot of cryopreserved HCAECs were from a single donor). All experiments were performed between passages 2 and 6. MDA-MB-231 cells (ECACC, cat: 9202042A) were maintained in DMEM supplemented with penicillin (250 units/ml), streptomycin (100 μg/ml), L-glutamine (27 mg/ml) and foetal calf serum at 10% (*v*/v). Cells were utilised between passages 10 and 20. All cells were kept in a humidified atmosphere containing 5% CO_2 at 37°C.

2.3. Western blotting

Proteins were separated by 10% SDS–PAGE and transferred onto nitrocellulose. The membranes were blocked for non-specific binding for 2 h. in 2% Bovine serum albumin (*w*/*v*) diluted in a sodium tris-tween (NATT) buffer (50 mM Tris–HCl, 150 mM NaCl, 0.02% (*v*/v) Tween-20). The blots were then incubated overnight with 50 ng/ml primary antibody diluted in 0.2% BSA (*w*/*v*) in NATT buffer. The blots were washed with NATT buffer for 90 min and incubated with HRP-conjugated secondary antibody (20 ng/ml in 0.2% BSA (w/v) diluted in NATT buffer) for 2 h. After a further 90 min wash, the blots were subjected to enhanced chemiluminescence reagent and exposed to Kodak X-ray film.

2.4. JNK activity assay

To measure JNK activity, cells were stimulated as appropriate and the reaction terminated by rapid aspiration and the cell monolayer washed with ice-cold PBS. The cells were solubilised in 20 mM HEPES buffer, pH 7.7, containing 50 mM NaCl, 0.1 mM EDTA, 0.1 mM Na_3VO_4, 0.1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% (*w*/*v*) Triton X-100. Lysates were clarified by centrifugation for 5 min at 13,000 rpm and equal amounts of protein were incubated with 20 μg of GST-c-Jun-(5–89) immobilized on glutathione-Sepharose at 4 °C for 3 h. Beads were then washed three times in solubilisation buffer and twice in 25 mM HEPES buffer, pH 7.6, containing 20 mM β-glycerophosphate, 0.1 mM NaV_3O_4, 2 mM dithiothreitol. Precipitates were then incubated with the same buffer containing 25 μM/1.85×10^{10} Bq of ATP/[$\gamma-^{32}$P] ATP in a final volume of 30 μl at 30 °C for 30 min. The reactions were terminated by the addition of 4 x SDS-sample buffer and aliquots of each sample subjected to electrophoresis on 11% SDS-PAGE. Phosphorylation of GST-c-Jun was then determined by autoradiography.

2.5. ELISA

To measure E-selectin expression, HUVECs were stimulated as appropriate and the reaction terminated by rapid aspiration and the cells solubilised in 20 mM HEPES buffer, pH 7.7, containing 50 mM NaCl, 0.1 mM EDTA, 0.1 mM Na_3VO_4, 0.1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% (w/*v*) Triton X-100. Lysates were clarified by centrifugation and supernatants used immediately or stored at -20 °C. ELISAs were performed according to manufactures guidelines (Human sE-selectin ELISA kit - Invitrogen). Briefly, micro-wells coated with an anti-E-selectin monoclonal antibody were washed prior to the addition of sample diluent, and standard dilutions were prepared in duplicate through a range of serial dilutions (1.6 ng/ml to 50 ng/ml). Cell supernatant samples were added in duplicate and diluted 1:5 with sample diluent and mixed thoroughly. An HRP-conjugated anti-E-selectin antibody was then added to all micro-wells and strips incubated for 2 h. shaking at room-temperature. Any unbound antibody was removed by a wash step prior to the addition of a HRP-reactive substrate solution; reactions were terminated by the addition of 500 mM phosphoric acid and absorbance measured at 450 nm on a BMG labtech, Polarstar Omega reader. The concentration of E-selectin present was determined using the standard curve generated.