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

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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 tri-phosphate (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.

Keywords: G-protein coupled receptors; Interleukin-1β; cC-Jun N-terminal kinase; pPurinergic receptors; iInflammation

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)

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

1.1 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].

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.

2.2 Material and Mmethods

2.1.2.1 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).

2.2.2.2 Cell Cculture

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 ug µg/ml ascorbic acid and 0.2 ug 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.

2.3.2.3 Western blotting

Proteins were separated by 10% SDS–PAGE and transferred onto nitrocellulose. The membranes were blocked for non-specific binding for 2 hrh. 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 hrh. After a further 90 min wash, the blots were subjected to enhanced chemiluminescence reagent and exposed to Kodak X-

ray film.

2.4.2.4 JNK Aactivity Aassay

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 hrh. 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 x x 10^{10} Bq of ATP/ [γ ^{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.

2.5.2.5 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 hrh. 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.

2.6.2.6 Data analysis and Sstatistical procedures

Where experimental data is shown as a blot, this represents one of at least 4 independent experiments and densitometry data represents the mean ± SEM. Statistical analysis was performed using Graph-pad prism 6.0, using one-way ANOVA with Dunnett's post-test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). EC_{50} and IC_{50} data was generated on Graph-pad prism 6, using a sigmoidal non-linear regression curve with variable slope analysis.

3.3 Results

3.1.3.1 ATP inhibits cytokine-driven JNK activity

Initially we examined the potential for several activators of endothelial cell GPCRs to mediate inhibition of JNK signalling. This included utilising trypsin, an endogenous activator of PAR2 [24], ATP/UTP endogenous activators of the purinergic receptors and histamine and endothelin-1. Of these agonists we found that ATP was by far the most efficacious.

Figure 1 panel A shows a concentration-dependent inhibition of IL-1β driven JNK activation, as mediated by ATP. Treatment with IL-1β (10 ng/ml) alone caused a 5–6 fold increase in JNK as assessed by either Western blotting for phospho-JNK or JNK activity as measured by in vitro kinase assay. Pre-treatment with increasing concentrations of ATP (1–100 μM) for 30 min substantially reduced cytokine mediated JNK signalling by approximately 70% (kinase = % stim, IL-1β = 100%, ATP (100 μM) + IL-1β =31.11 ± 12.73%, P<0.05). Whilst pre-incubation substantially inhibited the response, co-incubation with ATP (30 μM) and IL-1β (10 ng/ml) at 30 min was less effective (Fold stim – IL-1β = 10.75 ± 0.61, ATP + IL-1β = 6.68 ± 0.86, P<0.01), suggesting a minimal pre-incubation time is required to elicit a significant JNK inhibitory response (Fig. 1B).

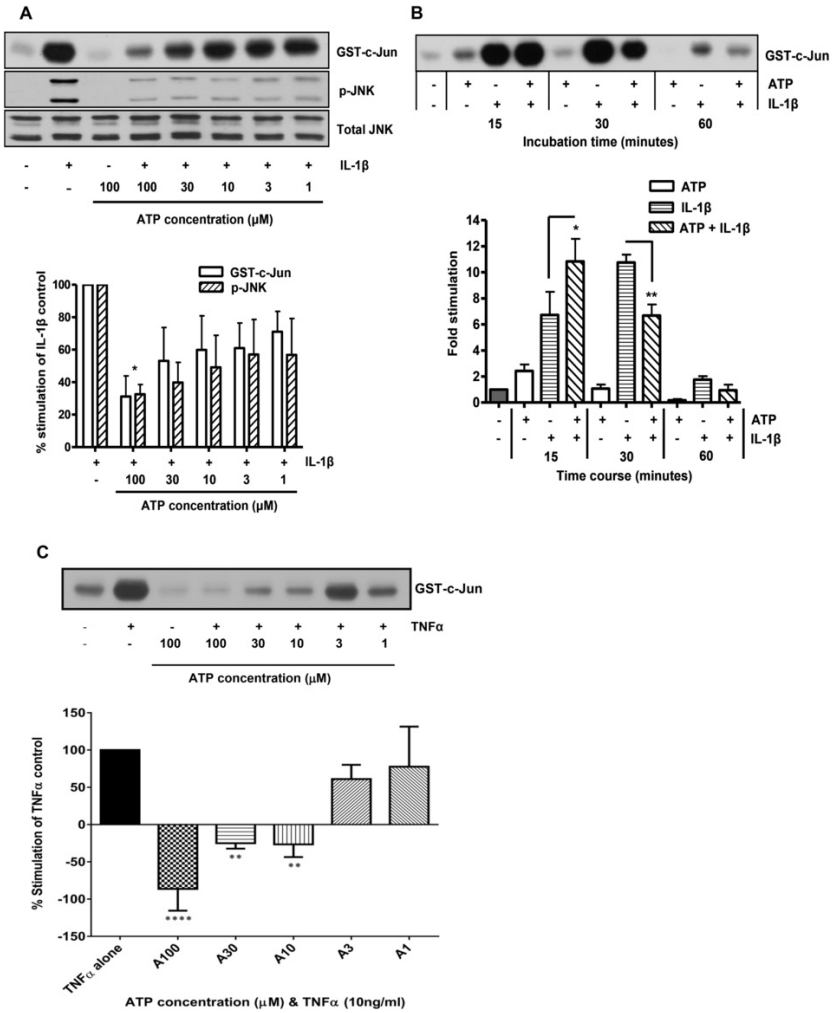

Fig. 1 ATP mediated inhibition of IL-1β-stimulated JNK phosphorylation and activity in HUVECs.

In Fig. 1A HUVECs were pre-incubated for 30 min with increasing concentrations of ATP prior to stimulation with IL-1β (10 ng/ml) for a further 30 min. Samples were assayed for JNK activity and phosphorylation as outlined in the methods section. In Fig. 1B HUVECs were treated simultaneously with ATP (100 μM) and IL-1β (10 ng/ml) for 15, 30 and 60 ~~minutes~~min. In Fig. 1C HUVECs were pre-incubated for 30 min with increasing concentrations of ATP prior to stimulation with TNFα (10 ng/ml) for a further 30 min. Samples were assayed for JNK activity. Proteins were quantified from JNK activity assays (Fig. 1B, C), and both JNK assays and phospho-blotting (Fig. 1A). Each value represents the mean ± SEM from 4 independent experiments and data was quantified by densitometry. *P <~~0~~.05, **P <~~0~~.01, ****P <~~0~~.0001 compared to IL-1β or TNFα alone.

alt-text: Fig. 1

A similar but more potent effect to that seen in panel A was observed when TNFα was used as the stimulus in HUVECs (Fig.1C). Where we observed a concentration-dependent inhibition of JNK activity ($IC_{50} = 7.04$ μM) with as little as 10 μM ATP abolishing all JNK activity in HUVECs (% stim, TNFα = 100~~.~~%, ATP (10 μM) + TNFα = ~~-~~26.48 ± 17.06~~.~~%, P<~~0~~.01).

~~3.2.~~3.2 Specificity of inhibition

The specificity of the inhibitory effect was further examined in Figu~~re~~. 2.~~.~~ In contrast to effects upon IL-1β, ATP was ineffective against either sorbitol, or anisomycin, two non-specific cellular stresses, both of which strongly

activated JNK (kinase: fold stim – sorbitol ~ 20 fold, anisomycin ~ 22 fold), similar to IL-1β (kinase: fold stim – IL-1β ~ 16 fold) (Fig. 2A). Furthermore, the effect of ATP was specific to cytokine –mediated JNK signalling. Whilst IL-1β was able to stimulate the other stress-activated protein kinase p38 MAP kinase (Fold stim – IL-1β = 5.42 ± 0.68 vs. control) this activity was not significantly affected by ATP (Fold stim - ATP (100μM) + IL-1β = 4.8 ± 0.38) as shown in Figure 2 panel B.

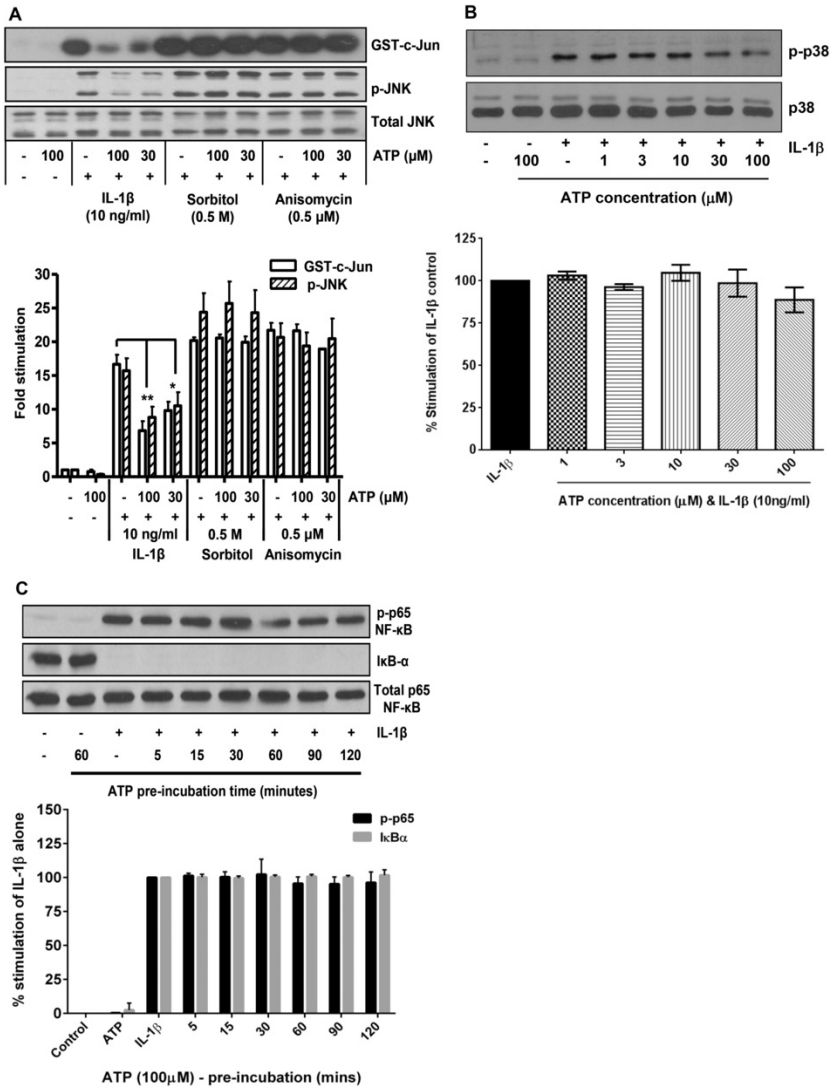

Fig. 2 Specificity of ATP mediated JNK inhibition in HUVECs.

In Fig. 2A HUVECs were pre-incubated for 30 min with 30 and 100 μM ATP prior to stimulation with IL-1β (10 ng/ml), sorbitol (0.5 M) and anisomycin (0.5 μM) for a further 30 min. Samples were assayed for JNK activity and phosphorylation. In Fig. 2B, HUVECs were incubated with increasing concentrations of ATP (1–100μM) for 30 min prior to stimulation with IL-1β (10 ng/ml) for a further 30 min. Phosphorylation of p38 MAPK was assessed and quantified as described in the methods section. In Fig. 2C, HUVECs were pre-treated with ATP (100 μM) for increasing time periods from 5 minutes min up to 120 minutes min, before stimulation with IL-1β (10 ng/ml) for 30 minutes min. ATP treatment alone at a middle time point (60 mins) was used as a control to show that receptor activation alone does not induce JNK activity. Samples were assayed for p65 phosphorylation, IκBα degradation and proteins quantified as outlined in the methods section.

Proteins were quantified from both JNK activity assays and phospho-blotting (Fig. 2A). Each value represents the mean ± SEM from at least 4 independent experiments and data was quantified by densitometry. *P<0.05, **P<0.01 compared to IL-1β alone.

alt-text: Fig. 2

This was also similarly demonstrated in our previous publications for both PAR2 and P2Y receptors [14, 16]. In addition, ATP was found to have no effect upon either IL-1β stimulated loss in cellular IκBα nor the phosphorylation of p65 (Ser536) (Fig. 2C), two well recognised markers of canonical NFκB activation [25, 26].

3.3.3.3 ATP inhibitory activity is not limited to HUVECs

We decided to examine this inhibitory phenomenon in other cells types, to eliminate the possibility that the effects observed in HUVECs were specific to this cell type. In Figure 3 panel A, we utilised another primary cell line, human coronary artery endothelial cells and similar to the HUVECs noted a concentration dependent inhibition of IL-1β - mediated JNK activity by ATP. Using 100 μM ATP for 30 minutesmin pre-treatment we demonstrated approximately 45% inhibition of IL-1β-mediated JNK kinase activity and approximately 40% inhibition of JNK phosphorylation (% stim, IL-1β = 100%, kinase = ATP (100 μM) + IL-1β = 54.4 ± 6.4%, P<0.01 and pJNK = ATP (100 μM) + IL-1β = 60 ± 5.9% P <0.01).

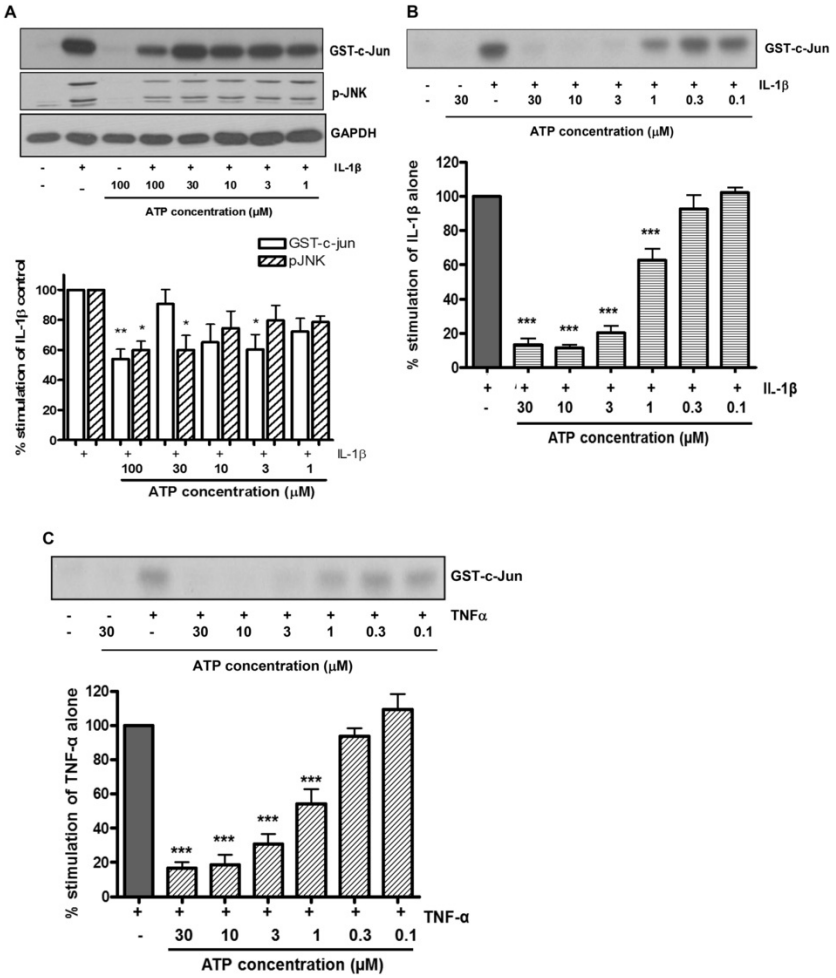

Fig. 3 ATP mediated inhibition of cytokine-stimulated JNK phosphorylation and activity is also evident in HCAEC and MDA-MB-231 cells.

In Fig. 3A HCAECs were pre-incubated for 30 min with increasing concentrations of ATP prior to stimulation with IL-1β (10 ng/ml) for a further 30 min. Samples were assayed for JNK activity and phosphorylation as outlined in the methods section. In Fig. 3B MDA-MB-231 cells were pre-incubated for 30 min with increasing concentrations of ATP prior to stimulation with IL-1β (10 ng/ml) for a further 30 min and assayed for JNK activity. In Fig. 3C, MDA-MB-231 cells were pre-incubated for 30 min with increasing concentrations of ATP prior to stimulation with TNFα (10 ng/ml) for a further 30 min and assayed for JNK activity. Proteins were quantified from both JNK activity assays and phospho-blotting (Fig. 3A) and from JNK activity assays (Fig. 3B, C). Each value represents the mean ± SEM from at least 5 experiments and data was quantified by densitometry. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to IL-1β or TNFα alone.

alt-text: Fig. 3

In Figure 3 panel B we used the human breast adenocarcinoma line MDA-MB-231 as an example of a homogenous cell population to use as a comparative to the primary cells when examining levels of inhibition. Here we also observed a significant concentration-dependent inhibition, with 30 μM ATP giving an 87% reduction in IL-1β-driven JNK activity (% stim, IL-1β = 100%, ATP (30 μM) + IL-1β =13.28 ± 3.6%, $p<0.001$). Inhibition was observed as low as 1 μM ATP where a 37% reduction was still evident before loss of inhibition at 0.3 μM ATP. This was true also when TNFα was used as the stimulus in the MDA-MB-231 cells (Fig. 3C) where we again observed a significant concentration-dependent inhibition. Using 30 μM ATP we observed an 83% reduction in TNFα-driven JNK activity (% stim, TNFα = 100%, ATP (30 μM) + TNFα =16.81 ± 3.48%, $P<0.001$) and concentrations as low as 1 μM ATP again showed significant inhibition of JNK activity, with approximately 46% reduction.

3.4.3.4 P2YR subtypes

Endothelium expresses a range of purinergic receptors including $P2Y_1$, $_2$ and $P2Y_{11}$ and the role of these subtypes in the inhibitory effect of ATP was examined in Figure 4. In the absence of a suitable $P2Y_2$ antagonist we tested the effect of UTP, a selective $P2Y_2$ agonist and found no significant inhibition of IL-1β-stimulated JNK thereby suggesting this receptor was not involved (data not shown). In addition, the $P2Y_1$ antagonist MRS2179 was without effect on either JNK activity or phosphorylation as shown in Figure 4, panel A. In contrast, in Figure 4 panel B, we found that pre-incubation with 10 μM of the $P2Y_{11}$ antagonist NF340 almost completely reversed the inhibitory effect of ATP on IL-1β-induced JNK signalling (% stim, IL-1β = 100%, ATP + IL-1β = 20.4% ± 10.8% and 10 μM NF + ATP + IL-1β = 87% ± 8.1%).

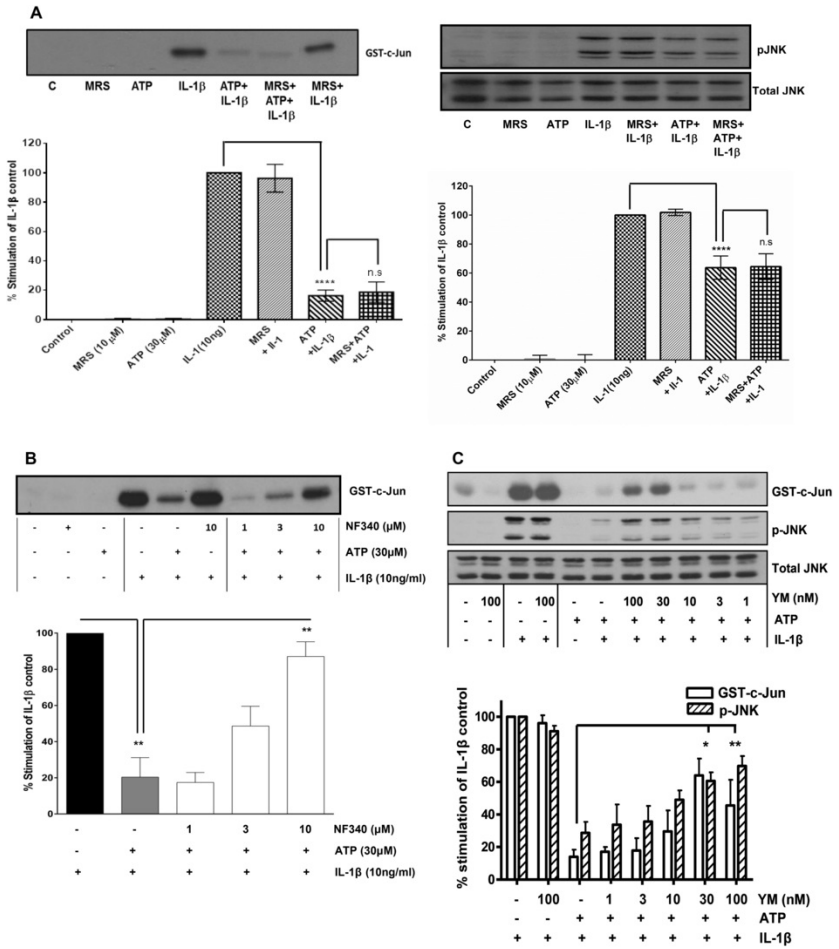

Fig. 4 ATP mediated inhibition of IL-1β-stimulated JNK phosphorylation and activity in HUVEC is via $P2Y_{11}$.

In Fig. 4A, HUVECs were pre-incubated with the P2Y1 antagonist MRS2179 (10 μM) for 30 min, followed by ATP (30 μM) for 30 min, and lastly IL-1β (10 ng/ml) for 30 min. In Fig. 4B, HUVECs were treated with increasing concentrations of the P2Y11 antagonist NF340 prior to stimulation with 30 μM ATP for 30 min followed by stimulation with IL-1β (10 ng/ml) for a further 30 min. In Fig. 4C, HUVECs were pre-incubated for 30 min with increasing concentrations of the Gαq/11 inhibitor YM254890 prior to stimulation with 30 μM ATP for 30 min followed by IL-1β (10 ng/ml) for a further 30 min. JNK activity was assessed by in vitro kinase assay (Fig. 4A, B, C) and phospho-blotting (Fig. 4A & C) as outlined in the methods section. Statistical analysis was performed using one way ANOVA with Dunnett's post-test. *P <~~0~~.05, **P <~~0~~.01, ****P < 0.0001 compared to IL-1β alone, n.s – not significant when compared to ATP and IL-1β. Each value represents the mean ± SEM from 4 independent experiments and data was quantified by densitometry.

alt-text: Fig. 4

In order to further confirm the actions of ATP in mediating JNK inhibition via the $P2Y_{11}$ receptor, we investigated further the signalling mechanisms involved. The $P2Y_{11}$ receptor couples to $G\alpha_{q/11}$ to mediate downstream signalling events; therefore we examined the role of $G\alpha_{q/11}$ as shown in Figure 4 panel C. Pre-incubation of HUVECs with the $G\alpha_{q/11}$ inhibitor YM254890 partially reversed the inhibition by ATP of IL-1β-induced JNK activity and phosphorylation at 30 and 100 nM YM by approximately 45%.

~~3.5.~~3.5 ATP-mediated inhibition of cytokine-stimulated JNK activation - specificity of $P2Y_{11}$

The results from Figure 4, examining inhibition of $G\alpha_{q/11}$, suggested that another G-protein dependent subunit may play a role in ATP-mediated inhibition of JNK. Indeed $P2Y_{11}$ couples to both $G\alpha_{q/11}$ and $G\alpha_s$ to mediate its

effects; therefore we investigated the effect of adenosine which strongly stimulates the A_{2A} and A_{2B} receptors via $G\alpha_s$ coupling to activate JNK in HUVECs. In Figure 5 panel A, we found that pre-treatment with adenosine (1–100 μM) lead to a concentration dependent inhibition of IL-1β-mediated JNK signalling, with approximately 68% inhibition evident using 100 μM adenosine (% stim, IL-1β = 100%, adenosine+IL-1β = 32.13% ± 12.21%, $P<0.01$) with an IC_{50} value of approximately 15 μM (15.41 ± 5.15 μM). Pre-incubation with the A_{2A} receptor antagonist ZM241385 (10μM), abolished the inhibitory effect of adenosine (% stim, IL-1β = 100%, adeno+IL-1β = 17.9% ± 6.7%, ZM+adeno+IL-1β = 103% ± 1.6%, P <0.01), but was without effect against ATP (Fig. 5B).

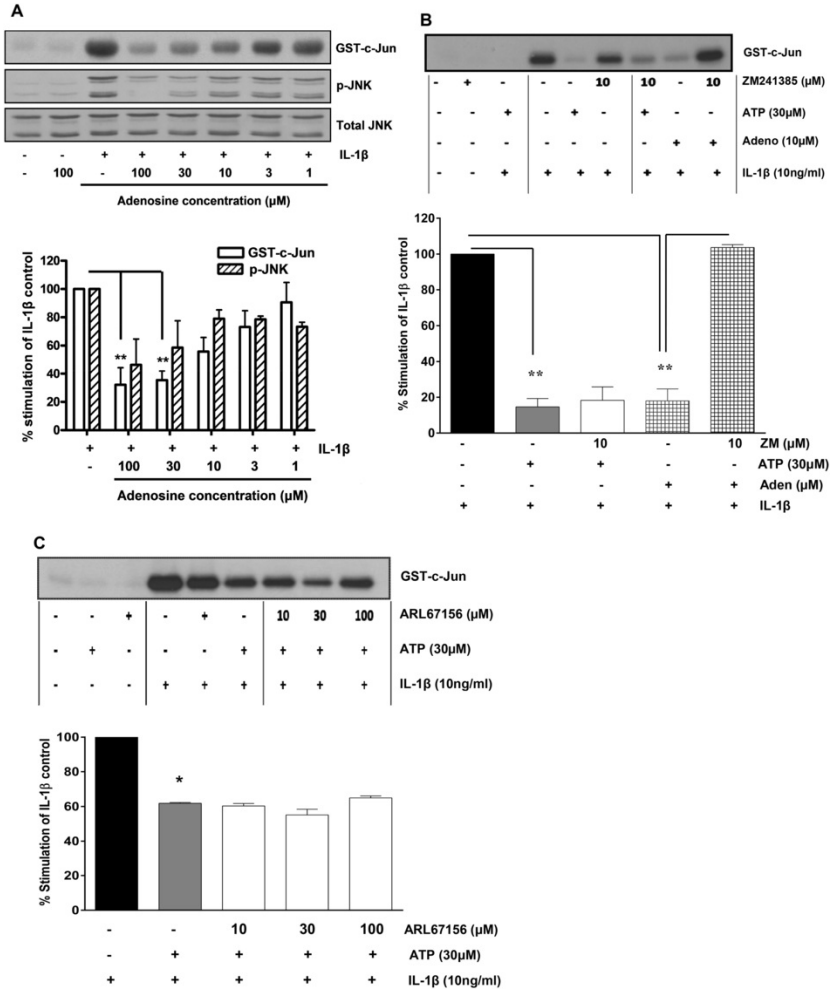

Fig. 5 ATP mediated inhibition of IL-1β-stimulated JNK activation in HUVECs does not involve the adenosine A2 receptor.

In Fig. 5A, HUVECs were pre-incubated for 30 min with increasing concentrations (1–100 μM) of adenosine, followed by stimulation with IL-1β (10 ng/ml) for a further 30 min. In Fig. 5B, HUVECs were treated with the A2A receptor antagonist ZM241385 (10 μM) for 30 min prior to stimulation with 30 μM ATP or 10 μM adenosine for 30 min followed by stimulation with IL-1β (10 ng/ml) for a further 30 min. In Fig. 5C, HUVECs were treated with increasing concentrations (10–100 μM) of the ecto-nucleotidase inhibitor ARL67156 prior to stimulation with 30 μM ATP for 30 min followed by stimulation with IL-1β (10 ng/ml) for a further 30 min. Samples were assayed for JNK activity (Fig. 5A, B & C) and phosphorylation (Fig. 5A) as outlined in the methods section. Proteins were quantified from both JNK activity assays and phospho-blotting. Each value represents the mean ± SEM from 5 independent experiments and data was quantified by densitometry. $^{**}P<0.01$ compared to IL-1β alone.

alt-text: Fig. 5

Another possibility was that ATP may mediate its action partially through the activation of the adenosine A2 receptor due to degradation of ATP via the ecto-nucleotidase. The experiment in Figure 5 panel C however shows that

pre-treatment of cells with the ecto-nucleotidase inhibitor, ARL67156 did not result in the reversal of the inhibitory effect of ATP, which further suggests that this mechanism was not involved.

~~3.6.~~3.6 G-protein coupling of $P2Y_{11}$

As $P2Y_{11}$ couples to both $G\alpha_{q/11}$ and $G\alpha_s$ to mediate its effects we decided to further dissect the contribution of each pathway to the overall inhibition as shown in Figure 6. The role of a further $G\alpha_s$ intermediate, PKA was assessed using the pharmacological inhibitor of PKA, namely H89. Pre-treatment of the cells with 10 μM H89 (Fig. 6A) also partially reversed the effect of ATP by approximately 36% (% stim, IL-1β = 100%, ATP + IL-1β = 44.42% ± 5.32%, H89 + ATP + IL-1β = 80.24% ± 5.14%, $P<0.01$), similar to that seen with the $G\alpha_{q/11}$ inhibitor YM254890 (Fig. 4) indicating a possible contribution from $G\alpha_s$-mediated PKA signalling also.

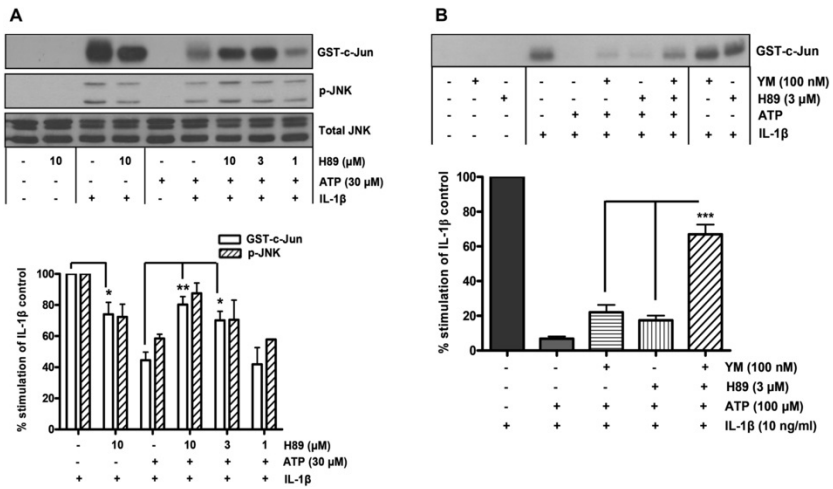

Fig. 6 $G\alpha_s$ dependent mechanisms also play a role in regulating IL-1β-stimulated JNK phosphorylation and activity in HUVECs.

In Fig. 6A, HUVECs were treated with increasing concentrations of the PKA inhibitor H89, for 30 min prior to pre-treatment with 30 μM ATP for a further 30 min followed by stimulation with IL-1β (10 ng/ml) for 30 min. In Fig. 6B, HUVECs were treated with 100 nM YM254890 alone or in combination with 3 μM H89 prior to stimulation with 30 μM ATP for 30 min followed by stimulation with IL-1β (10 ng/ml) for a further 30 min. Samples were assayed for JNK activity (Fig. 6A & B) and phosphorylation (Fig. 6A) as outlined in the methods section. Proteins were quantified from both JNK activity assays and phosphoblotting. Each value represents the mean ± SEM from at least 4 independent experiments and data was quantified by densitometry. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to IL-1β alone.

alt-text: Fig. 6

The H89 compound however also appeared to be mediating an effect on IL-1β-mediated JNK activation alone, lowering the response by approximately 20% (Fig. 6A). Treatment with the H89 compound did appear to partially reverse ATP-mediated inhibition, as the response was brought back to that of H89 and IL-1β alone (not significant). The reversal was generally greater when the cells were pre-incubated with both compounds in combination (100 nM YM254890 and 3 μM H89), where a partial reversal of approximately 60% was evident compared to either 16% reversal for YM2548909 or 11% reversal for H89 alone (Fig. 6B).

~~3.7.~~3.7 JNK mediated downstream translational responses

We further sought to determine if inhibition of JNK by ATP was applicable to any cellular event relevant to endothelial cell pathophysiology. Figure 7 shows the effect of ATP pre-treatment on IL-1β-mediated induction of COX-2 and the adhesion molecules, ICAM-1 and VCAM-1. IL-1β stimulated a concentration-dependent increase in COX-2 expression (Fig. 7A) which reached maximum (approximately 8.5 fold increase over basal) using 10 ng/ml of IL-1β for 8 ~~hr~~h.

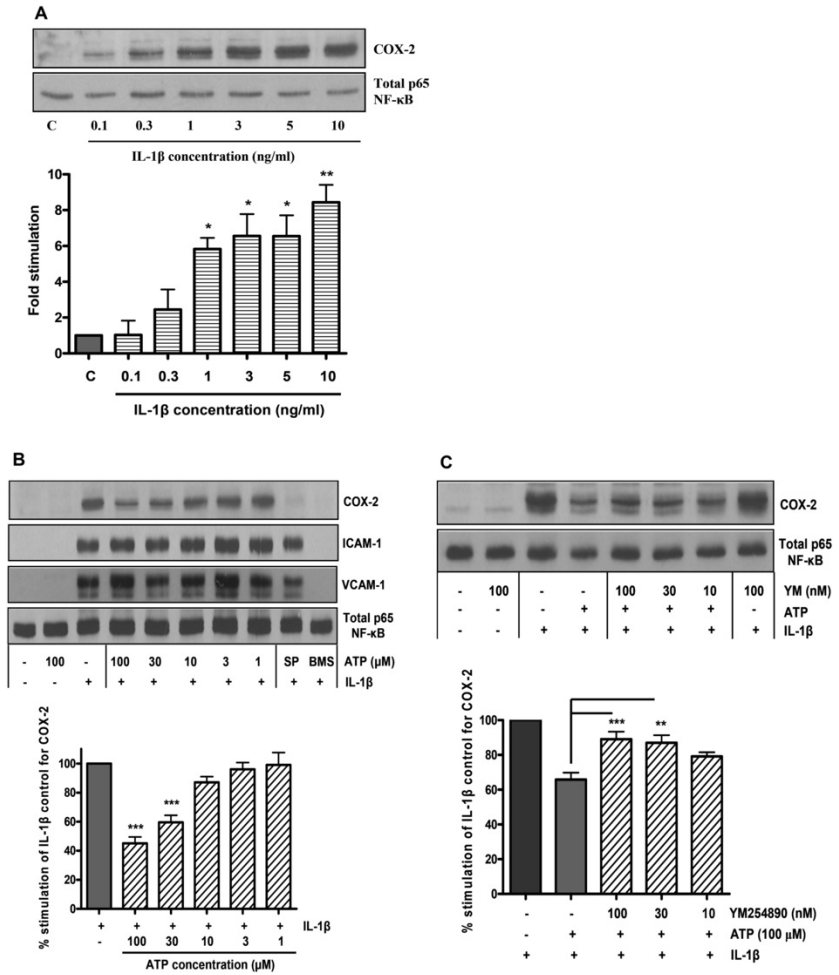

Fig. 7 ATP mediated inhibition of COX-2 expression in HUVECs.

In Fig. 7A, HUVECs were treated with increasing concentrations of IL-1β from 0.1 to 10 ng/ml for 8 ~~hr~~h and COX-2 expression assessed. In Fig. 7B, HUVECs were pre-incubated with increasing concentrations of ATP prior to stimulation with IL-1β (10 ng/ml), for a further 30 min, JNK inhibitor SP600125 (20 μM) and NFκB inhibitor BMS-345541 (10 μM) were used as positive controls and added 30 min prior to IL-1β. Samples were assayed for COX-2, ICAM-1, and VCAM-1 expression. In Fig. 7C, HUVECs were pre-treated with increasing concentration of the Gαq/11 inhibitor YM254890 for 30 min prior to stimulation with 30 μM ATP for 30 min, followed by stimulation with IL-1β for a further 30 min. Samples were assayed for COX-2 expression and NFκB-p65 as a loading control as outlined in the methods section. Gels were quantified for protein expression (Fig. 7A, B & C). Each value represents the mean ± SEM from at least 4 experiments and data was quantified by densitometry. $^{**}P<$ ~~0~~.01, $^{***}P<$ ~~0~~.001 compared to IL-1β alone.

alt-text: Fig. 7

In Fig~~ure~~. 7 panel B, pre-treatment of the cells with 100 μM ATP reduced the expression of COX-2 by approximately 60% (% stim, IL-1β = 100%, ATP + IL-1β = 45.05% ± 4.39%, $P<$ ~~0~~.001), whilst the JNK inhibitor, SP600125 completely abolished expression, confirming JNK regulation of COX-2 expression. In contrast, ATP or SP600125 did not affect IL-1β induced expression of ICAM-1 and VCAM-1, two adhesions molecules not known to be regulated by JNK but rather NFκB, thereby confirming the effect of ATP is selective to the JNK pathway. The expression of ICAM-1 and VCAM-1 as well as COX-2 were however sensitive to BMS-34551, an inhibitor of inhibitory kappa B kinases (Fig. 7B).

In Fig~~ure~~. 7 panel C we show that pre-incubation with 100 nM of the $G\alpha_{q/11}$ inhibitor YM254890 was able to partially reverse the inhibitory effect of ATP on IL-1β-induced COX-2 expression by approximately 25% (% stim, IL-1β =

100%, ATP + IL-1β = 65.78% ± 3.99%, YM + ATP + IL-1β = 89.08% ± 4.28%, P < 0.001). However, when the PKA inhibitor H89 was used in similar types of experiment a near complete reversal of IL-1β-induced COX-2 expression was observed with H89 alone, suggesting a prominent role for $G\alpha_s$ (data not shown).

3.8.3.8 ATP inhibits IL-1β-driven E-selectin expression

In addition we also examined another JNK dependent cellular protein, E-selectin which is expressed predominantly on the cell membrane and plays a key role in inflammation. In Figure 8 we demonstrate the effect of ATP pre-treatment on the expression of E-selectin. IL-1β was shown to stimulate a strong concentration- and time-dependent increase in E-selectin expression in HUVECs with maximal expression at 4 h post treatment (data not shown). We show in Figure 8 panel A, that pre-treatment of the cells with ATP reduced the expression of E-selectin in response to IL-1β by approximately 44% (IL-1β = 24.9 ng/ml ± 1.7, ATP + IL-1β = 13.3 ng/ml ± 1.5). Interestingly, the JNK inhibitor, SP600125 also reduced IL-1β-mediated E-selectin expression by approximately 44% (SP + ATP + IL-1β = 13.4 ng/ml ± 1.0), thus suggesting that ATP was indeed inhibiting JNK-mediated expression of E-selectin in HUVECs (Fig. 8A).

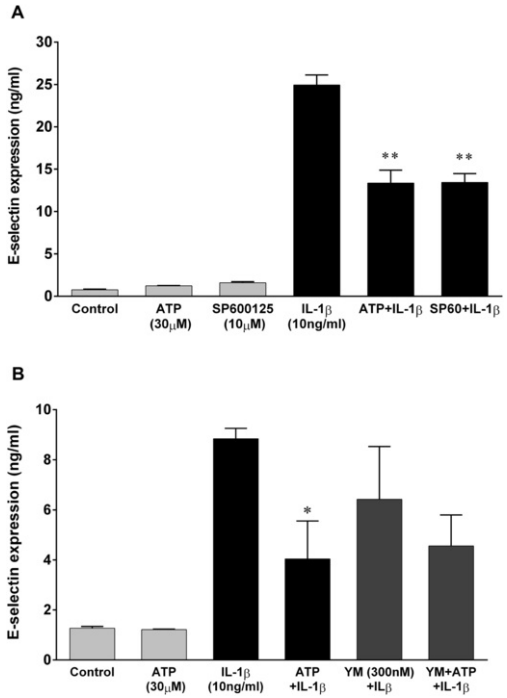

Fig. 8 IL-1β induction of E-selectin is regulated by ATP in HUVECs.

In Fig. 8A, HUVECs were treated with the JNK inhibitor – SP600125 (10 μM) for 120 min alone, or 30 min prior to pre-treatment with 30 μM ATP for a further 30 min, cells were then stimulated with 10 ng/ml of IL-1β for 60 min. Cells were lysed and supernatants assayed for E-selectin expression. In Fig. 8B, HUVECs were treated with the Gαq/11 inhibitor YM254890 (100 nM) for 30 min prior to pre-treatment with ATP (30 μM) for a further 30 min, this was followed by stimulation with IL-1β (10 ng/ml) for 60 min. Cells were lysed and supernatants assayed for E-selectin expression as outlined in the methods section. Results were graphed as expression of E-selectin (ng/ml) as determined from a standard curve. Results are representative of 6 independent experiments, data calculated as mean ± SEM, $*P < 0.05$, $**P < 0.01$ compared to IL-1β alone.

alt-text: Fig. 8

In Figure 8 panel B, the effect of the $G\alpha_{q/11}$ inhibitor, YM254890 on ATP mediated inhibition of E-selectin was examined. While again, ATP was consistent in reducing IL-1β-mediated E-selectin expression (approximately 80% inhibition from IL-1β alone), $G\alpha_{q/11}$ did not appear to have a role in reversing this effect. The YM compound did however have a small effect on IL-1β-mediated E-selectin expression alone (IL-1β = 8.8 ng/ml ± 0.4, YM + IL-1β = 6.4 ng/ml ± 2.1), although this was not significant it suggests the potential that $G\alpha_{q/11}$ pathways may mediate IL-1β signalling in endothelial cells.

4.4 Discussion

In this study we describe the potential for ATP to inhibit cytokine-mediated signalling in primary cultures of endothelial cells. Indeed pre-treatment with ATP reduced cytokine-mediated signalling in a time- and concentration-dependent manner mediated principally via the $P2Y_{11}$ receptor and did not involve degradation to adenosine as part of the inhibition mechanism. Our findings support and extend our previous studies which highlight the potential for receptors linked to $G\alpha_{q/11}$ to mediate a selective inhibition of JNK signalling but also indicated $G\alpha_s$ dependent signalling maybe involved. We also demonstrated for the first time for this phenomenon to be relevant to JNK dependent outcomes pertinent to inflammation, namely COX-2 and E-selectin, which are involved in the pathophysiology of the endothelium.

Within the primary endothelial cell environment, we believe varying levels of receptor expression across cell passages and batches used for the duration of the study may be the reason for the slight inconsistency seen in the levels of inhibition throughout these studies; this was true also when using the HCAECs. In comparison levels of inhibition observed using a standard cell line MDA-MB-231 remained consistent. Receptor expression was not measured directly as $P2Y_{11}$ antibodies have been shown to lack specificity, and mRNA expression in many cases does not correlate to protein expression [27]. Despite these variations within the primary endothelial cells, the salient point was that statistically significant inhibition was always evident with ATP pre-treatment in all experiments.

A key consideration to this study is the levels of ATP present within the endothelium. It is well recognised that endothelial cells release substantial quantities of ATP during injury [28] and inflammation [29, 30]. With ATP at the endothelial surface reported to be approximately 14 μM [31]. However further studies have demonstrated that concentrations of extracellular ATP released from injured cells could reach 125 μM at the moment of cell injury [32]. Similarly, a study by Pellegatti and colleagues measuring extracellular ATP in vivo have reported concentrations in the hundreds micromolar range in tumour interstitium [33]. Thus such levels are consistent with the concentrations required to initiate inhibition in this study.

The inhibitory actions of ATP was specific to cytokine mediated JNK activation rather than a general inhibition of JNK per se, as pre-treatment had no effect upon stress induced JNK activation mediated by sorbitol and anisomycin. This suggests a phenomenon consistent with previous findings from our laboratory but also complements a previous study which shows that activation of the histamine H1 receptor was able to inhibit TNFα- and thrombin-mediated activation of both p38 MAP kinase and JNK pathways [34]. In addition, other GPCRs such as the cannabinoid CB2 receptor have also been shown to inhibit cytokine mediated JNK activity in human coronary artery smooth muscle cells [35]. In this present study we show inhibition to be largely specific to JNK suggesting cell type specific differences in GPCR driven inhibition of cytokine mediated activation of MAPK pathways.

Further characterisation revealed a role for $P2Y_{11}$ since the inhibitory response was sensitive to the $P2Y_{11}$ selective antagonist NF340, [17]. This is in contrast to our previous studies which implicated the involvement of $P2Y_2$ receptors in mediating inhibition of TNFα and sorbitol stimulated JNK and p38 MAPK activity [16]. Different endothelial cell types vary in their P2YR expression profiles with HUVECs predominantly expressing $P2Y_{11}$ receptors but additionally $P2Y_1$ and $P2Y_2$ [3]. We also tested $P2Y_1$ inhibition using the $P2Y_1$ antagonist MRS2179 and showed no effect on ATP mediated inhibition of IL-1β driven JNK activity. We decided to investigate treatment with MRS2179 alone or in combination with the $P2Y_{11}$ antagonist NF340, but did not observe any synergistic effects (data not shown). This is pertinent to recent studies that reveal dimerisation between $P2Y_1$ and $P2Y_{11}$ receptors are required for the internalisation of the $P2Y_{11}$ receptor [36].

As well as acting directly with cognate receptors, ATP can be degraded by a number of membrane bound ATPases resulting in the formation of metabolites such as ADP, AMP and adenosine [37]. Indeed we found that adenosine caused a concentration dependent inhibition of JNK suggesting this as a potential mechanism. Nevertheless, even though the recognised kinetics of ATP degradation, which is significant within 30 min [38], occurs well within the time span of the experiments in this study, neither the ectoATPase inhibitor nor the adenosine A2A inhibitor reversed the inhibition of JNK mediated by ATP, precluding this as a mechanism of activation.

We have previously demonstrated the involvement of $G\alpha_{q/11}$ in PAR2-mediated inhibition of TNFα -stimulated JNK phosphorylation and activation [14]. We demonstrated here for endogenously expressed $P2Y_{11}$ a similar dependency using the novel $G\alpha_{q/11}$ inhibitor YM254890 [23, 39]. However, unlike our previous study the reversal was only partial in response to IL-1β, suggesting the potential of a $G\alpha_{q/11}$ independent effect. $P2Y_{11}$ is linked to both $G\alpha_{q/11}$ and $G\alpha_s$ suggesting the possibility of the $G\alpha_s$ axis as an additional component. Indeed, adenosine in engaging the A_{2A} receptor mediates its effects via $G\alpha_s$ [40] and also in this study caused a significant inhibition of JNK. We found that the PKA inhibitor H89 was able to partially reverse ATP mediated inhibition suggesting an additional role for this pathway and one which may or may not predominate. This is in keeping with a number of studies which show that $G\alpha_s$ linked receptors can also mediate an inhibition of JNK signalling, for example, the Dopamine D2 receptor [41], but is in contrast to earlier studies linking $G\alpha_s$ coupled receptors to the activation of JNK [42].

A key additional component to this work was the potential for ATP mediated JNK inhibition to have consequences further downstream linked to the expression of inflammatory mediators. We demonstrated that ATP was able to inhibit the induction of COX-2 in response to IL-1β, an effect which could be almost completely reversed by $G\alpha_{q/11}$ inhibition. In contrast, the PKA inhibitor H89 alone abolished COX-2 induction suggesting possible off-target effects of this inhibitor, and making interpretation and deduction of the role of the $G\alpha_s$ pathway in COX-2 induction difficult. H89 has been shown to inhibit at least 8 other kinases [21], therefore further studies examining the role of $G\alpha_s$ would be required to explore this further.

A number of studies demonstrate that COX-2 expression in the endothelium is indeed dependent upon JNK [43–45] and in this current study we show that induction was sensitive to the JNK inhibitor SP600125. As a corollary

we tested effects on ICAM-1 and VCAM-1, two NFκB dependent genes [46] and found no ATP mediated inhibition suggesting that the inhibitory effect of ATP pre-treatment was consistent with JNK inhibition and not a general one. Whilst the role of COX-2 in endothelial cell inflammation is controversial, studies have demonstrated JNK dependent up-regulation of COX-2 in atherosclerotic lesions [47] proposed to mediate the activation of the EP4 receptor via prostaglandin E2 (PGE_2) release leading to secretion of inflammatory mediators [48, 49]. Our studies however imply a functional effect of COX-2, with induction in endothelial cells mediated by pro-inflammatory cytokines, but with the potential to be down-regulated via purinergic receptor signalling. COX-2 induction however, may also be cytoprotective and inhibition of expression may be deleterious to endothelial integrity [50, 51].

The role of E-selectin in endothelial cell inflammation is well established, for example the local release of cytokines such as IL-1β and TNFα by damaged cells induces the over-expression of E-selectin on endothelial cells of nearby blood vessels. This increased expression acts to mediate leukocyte adhesion and recruitment to sites of injury, which is also prevalent during shear stress of blood flow, leading to leukocyte rolling along the blood vessel walls [52]. Studies have demonstrated a role for endothelium-derived nitric oxide (NO) in inhibiting cytokine mediated expression of E-selectin, VCAM-1 and ICAM-1 via NFκB signalling, thereby preventing recruitment of leukocytes to the vessel wall thus suggesting another paradigm whereby the endothelium confers protection against inflammatory events [53].

Another study has shown a role for adenosine in inhibiting both cytokine release and expression of adhesion molecules, including E-selectin in HUVECs, however they could not demonstrate a clear role for adenosine receptors nor did they investigate ATP [54]. This study therefore is the first of its kind to demonstrate a role for ATP in inhibiting IL-1β-mediated E-selectin expression in human primary endothelial cells. This would be of particular importance to the endothelium, as a reduction in E-selectin expression would limit the recruitment of immune cells and associated pro-inflammatory mediator release, thereby reducing the damage at the endothelial surface.

5.5 Conclusions

This study develops further the initial phenomena uncovered, showing a role for GPCRs in modulating pro-inflammatory cytokine signalling. This also starts to reveal the functions of the $P2Y_{11}$ receptor which has been historically difficult to research and characterise fully due to a lack of murine models, effective antibody reagents to aid detection and functional methods [27]. Taken together these results highlight the importance of the purinergic receptors, in particular $P2Y_{11}$, in mediating a protective role against cytokine induced stress-activated signalling in the endothelium.

Indeed the potential for GPCRs to mediate inhibition of cytokine driven JNK signalling has a number of implications. It could be envisaged that ATP release mediated for example by endothelial distention or stretch could dampen down inflammation thus limiting the potential for initiation of plaque formation [55]. Thus, developing agonists which could activate endothelial cell GPCRs or directly activate either $G\alpha_{q/11}$ or $G\alpha_s$ may be of clinical potential in the context of inflammatory-based conditions.

Disclosures

Fig. 4A (left panel) the blot in this figure was cropped to omit 3 bands which consisted of combination stimulations using MRS and another inhibitor and controls, which we decided wasn't relevant to the point we were making here. Access to the full blot can be granted if requested.

Declarations of interest

None.

Author Ccontributions

Authors listed 1st (P.Y.N) and 2nd (K.A.M) contributed equally to the scientific work and as such are to be listed as joint first authorship. K.A.M wrote and structured the manuscript and is to be listed as corresponding author; G.H conducted the HCAEC experiments, analysed the data and produced the corresponding figure. K.H.H contributed to the culturing of the HUVECs and produced the p-p38 blots. A.P, R.P and K.A.M provided guidance in regards to experimental design and preparation of the manuscript. All authors have approved the manuscript.

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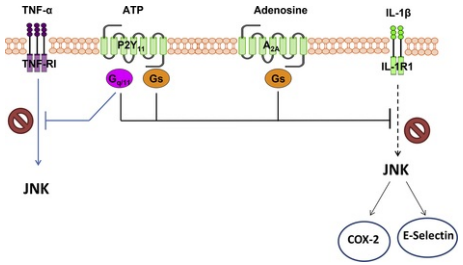
Graphical abstract



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