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Effects of adenosine A_{2A} and A_{2B} receptor activation on signalling pathways and cytokine production in the human urothelium

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ABSTRACT

Extracellular adenosine is formed in response to inflammation and the adenosine A_{2A} and A_{2B} receptor subtypes have both been implicated in modulation of inflammation. In the present study, we examined adenosine A_{2A} and A_{2B} receptor expression and signalling pathways in human uroepithelial cells. Modulation of adenosine A_{2A} and A_{2B} receptor activation on the uropathogenic *Escherichia coli* (UPEC)-stimulated IL-8 host response was also evaluated. The human uroepithelial cell line UROtsa was grown in cell culture and stimulated with a mixture of pro-inflammatory cytokines (CM) or UPEC. Receptor expression was examined by RT-PCR and IL-8, intracellular cAMP and phosphoproteins were measured by ELISA, EIA and multiplex immunoassay (Luminex), respectively. The mRNA expression of the adenosine A_{2A}, but not A_{2B}, receptor was up-regulated in response to CM and UPEC. The adenosine A_{2A} receptor agonist, CGS 21680 did not stimulate cAMP production but CREB phosphorylation was slightly increased. By contrast, the adenosine A₂ receptor agonist CPCA induced a pronounced cAMP and CREB response. Furthermore, adenosine A_{2A}, but not A_{2B}, receptor activation decreased ERK1/2, JNK, p38 and STAT3 phosphorylation. UPEC-infection stimulated the host IL-8 production but CPCA or CGS 21680 had no impact on basal or UPEC-evoked IL-8 production. In conclusion, our data identified marked differences in signalling pathways by activation of the adenosine A_{2A} and A_{2B} receptors. Activation of the adenosine A_{2A} receptor inhibited STAT3 and MAPK-signalling, while the cAMP-CREB pathway was induced by adenosine A_{2B} receptor activation. No anti- or pro-inflammatory effects were found for uroepithelial adenosine A_{2A} or A_{2B} receptors.

1. Introduction

Extracellular adenosine is produced from ATP during inflammation and tissue injury and is considered as a potent anti-inflammatory mediator (Hasko and Cronstein, 2004; Sitkovsky et al., 2004). The effect of adenosine is mediated by four G-protein coupled receptors, namely the adenosine A₁, A_{2A}, A_{2B} and A₃ receptor

subtypes (Fredholm et al., 2001). A prominent role for the adenosine A_{2A} receptor subtype in mediating the anti-inflammatory activities of adenosine is now well established. However, recent studies show that activation of adenosine A_{2B} receptors also exerts anti-inflammatory effects but there are some controversies whether the adenosine A_{2B} receptor subtype is anti- or proinflammatory (Linden, 2006).

During a urinary tract infection (UTI) uropathogenic *Escherichia coli* (UPEC) bind to uroepithelial cells that are triggered to

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produce proinflammatory mediators such as IL-6 and IL-8 as part of the host response (Svanborg et al., 2006). The uroepithelial cells lining the human urinary tract express the adenosine A₁, A_{2A} and A_{2B} receptor subtypes (Phelps et al., 2006; Save et al., 2009) but the role of these receptors in the mucosal inflammatory network is not understood. In other tissues, signalling via adenosine A_{2A} receptors inhibits inflammation (Ohta and Sitkovsky, 2001), in part by decreasing the release of pro-inflammatory cytokines (Bouma et al., 1994; Zidek, 1999). The anti-inflammatory characteristics of adenosine A_{2B} receptors also involve inhibition of cytokine production (Linden, 2006). Most of the anti-inflammatory effects of adenosine are thought to involve a rise in intracellular cAMP levels (Fredholm et al., 2007). Generally, activation of the adenosine A₁ and A₃ receptors leads to a decrease in intracellular cAMP levels by coupling to inhibitory G_{i/o} proteins. In contrast, adenosine A_{2A} and A_{2B} receptors couple to stimulatory G_s proteins and activate adenylyl cyclase, production of cAMP and activation of the PKA/CREB pathway (Fredholm et al., 2000). In addition, by couple to G_q proteins the adenosine A_{2B} receptor are able to activate phospholipase C (Feoktistov and Biaggioni, 1997). All the adenosine receptors have also been shown to activate at least one subgroup of the mitogen activated protein kinase (MAPK)-family, including ERK 1/2, p38 and JNK (Schulte and Fredholm, 2003).

The aim of this study was to examine whether UPEC-infection or cytokines influence adenosine A_{2A} and A_{2B} receptors expression and signalling pathways in human uroepithelial cells. Besides cAMP, other signalling pathways implicated in inflammation such as MAPK signalling, JAK-STAT signalling and NF- κ B signalling were also studied. In addition, we examined whether adenosine A_{2A} and A_{2B} receptor activation had an anti- or proinflammatory profile on the host IL-8 response.

2. Materials and Methods

2.1 Urinary tract epithelial cells and bacteria

The human urinary tract epithelial cell line, UROtsa, was kindly supplied by

Professor Scott Garrett (University of North Dakota, USA). The UROtsa cell line are derived from epithelial cells lining the ureter and immortalized by using SV-40 large T-antigen (Rossi et al., 2001). UROtsa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 U/ml penicillin and 100 μ g/ml streptomycin (all from Sigma Aldrich, St. Louis, MO, USA) at 37°C, 5% CO₂ in a humidified atmosphere and subcultured when confluent.

The uropathogenic *Escherichia coli* (UPEC) strain IA2 was originally isolated from a patient with acute pyelonephritis and express both P- and type 1-fimbriae. IA2 was cultured on tryptic soy agar plates (TSA) (Becton, Dickinson and Company, Sparks, MD, USA) at 37°C.

2.2 Cell stimulation procedure

For bacterial infection confluent monolayers of UROtsa cells were incubated with cell culture medium containing 10⁸ CFU/ml of UPEC strain IA2. Bacterial multiplication was limited by incubating the cells with gentamycin (50 μ g/ml) 24 hours prior to infection. Gentamycin was excluded during infections. UROtsa cells was also exposed to a mixture of proinflammatory cytokines (CM), IL-1 β (1 ng/ml), TNF α (1ng/ml) and IFN- γ (100U/ml) (Sigma-Aldrich). Cells incubated with medium alone were used as control.

2.3 RNA preparation, cDNA synthesis and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from UROtsa cells using RNeasy Minikit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. 1 μ g of total RNA was converted to cDNA using oligo-dT₁₆ primers (1 μ M) (Applied Biosystems, Foster City, CA, USA) and the Omniscript RT kit (Qiagen Inc.). RNA was controlled for genomic DNA contamination. PCR (25 μ l reaction volume) was performed using Ready-To-Go PCR beads (Amersham Biosciences, Buckinghamshire, UK). A volume of 2-5 μ l of the RT-reaction

was mixed with primers (0.5 μ M) for A_{2A}: forward 5'-AAC CTG CAG AAC GTC ACC A-3' and reverse 5'-GTC ACC AAG CCA TTG TAC CG-3' (245 bp), A_{2B}: forward 5'-GTG CCA CCA ACA ACT GCA CAG AAC-3' and reverse 5'-CTG ACC ATT CCC ACT CTT GAC ATC-3' (517 bp) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'-ATT CCA TGG CAC CGT CAA GGCT-3' and reverse 5'-TCA GGT CCA CCA CTG ACA CGT T-3' (571 bp) and PCR was performed in a thermal cycler (Perkin Elmer GeneAmp PCR system). PCR was run with an initial denaturation for one min at 94°C followed by 24-35 cycles at 94°C for one minute, 65°C for 30 seconds and one minute at 72°C, followed by a final extension at 72°C for 10 minutes for the adenosine A_{2A} receptor. For the adenosine A_{2B} receptor the initial denaturation was run for four minutes at 94°C followed by 24-35 cycles at 94°C for 45 seconds, 60°C for 45 seconds and two minutes at 72°C and a final extension at 72°C for seven minutes. PCR products were analysed by agarose gel (2%) electrophoresis and visualised by ethidium bromide staining. PCR products of GAPDH were diluted 1:10 before electrophoresis.

2.4 Determination of intracellular cAMP

Confluent UROtsa cells in 24-well plates (Sarstedt) were pretreated with CM or with IA2 (10⁸ CFU/ml) for 24 hours. The medium was removed and replaced with fresh medium and cells were stimulated with the adenosine A₂ receptor agonist CPCA (0.1, 1 and 10 μ M), the adenosine A_{2A} receptor agonist CGS 21680 (0.1 and 1 μ M) or the adenylyl cyclase activator forskolin (10 μ M) for 20 minutes. In some experiments UROtsa cells were exposed to IA2 for 1 hour. The medium was then removed and cells were lysed with 0.1 M HCl for 20 minutes at room temperature, collected by scraping and centrifuged at 600 x g for 10 minutes. Supernatant was collected, acetylated and used for measuring cAMP with the Direct cyclic AMP Correlate-EIA™ kit (Assay Designs, Inc., MI, USA). Optical density was measured at 405 nm in a Spectracount™ Packard AS 10001 fluorescence spectrophotometer.

2.5 Determination of intracellular phosphoproteins by Luminex

UROtsa cells were seeded in 24-well plates and when confluent normal medium was replaced with serumfree medium and cells were incubated for 4 hours. Thereafter UROtsa cells were stimulated with the adenosine A₂ receptor agonist CPCA (1 and 10 μ M), the adenosine A_{2A} receptor agonist CGS 21680 (0.1 and 1 μ M) for 7 or 15 minutes. Cells stimulated with EGF (50 ng/ml) were used as positive controls. Cells were lysed with lysis buffer (provided by the Luminex kit) supplemented with Complete mini, a protease inhibitor cocktail (Roche Diagnostics, Pleasanton, CA, USA) on an orbital shaker. The lysate was filtered in spin-x centrifugation tubes (0.22 μ m filter) (Sigma) at 14 000 x g for 1 minute and then stored at -70°C until analysis. Unstimulated cells were used as control. The protein concentrations of the samples were determined with the Bicinchoninic acid (BCA)™ protein assay kit (Pierce). Equal amounts of protein (5 μ g) were analysed for ERK 1/2, CREB, STAT3, STAT5, JNK, p70 S6 kinase, p38 and I κ B α with the MILLIPIX™ MAP 8-plex multi-pathway signalling kit (Millipore Corp., Billerica, MA, USA) according to manufacturer's instructions on a Luminex 200™ (Millipore). Data are expressed as mean fluorescence intensity (MFI).

2.6 Determination of IL-8 secretion

Confluent UROtsa cells grown in 24-well plates were exposed to 10⁶, 10⁷ and 10⁸ CFU/ml of UPEC strain IA2 for 6 and 24 hours or forskolin (10 μ M) for 24 hours. To determine the effect of adenosine A₂ receptor activation, UROtsa cells were exposed to IA2 (10⁸ CFU/ml) and after 2 hours of infection, cells were stimulated with the adenosine A_{2A} receptor agonist, CGS 21680 (0.1 and 1 μ M) (Sigma) or the adenosine A₂ receptor agonist CPCA (1 and 10 μ M) for an additional 22 hours. In some experiments cells were treated with the adenosine A_{2A} receptor antagonist, SCH 58261 (10 nM) (Sigma) 20 minutes prior to the addition of CPCA. Supernatants were centrifuged at 5000 x g for 7 minutes. Samples were placed at -20°C until IL-8 was analyzed with the BD OptEIA™ human IL-8 ELISA kit II (BD Biosciences Pharmingen Torreyana Rd., San Diego, USA). IL-8 was determined by measuring optical

Fig. 1. Adenosine receptor mRNA expression in UROtsa cells demonstrated by RT-PCR. Basal expression of adenosine A_1 , A_{2A} and A_{2B} receptor mRNA was detected, but transcript for the adenosine A_3 receptor was not found (A). Expression of adenosine A_{2A} and A_{2B} receptors in cytokine-stimulated (B and D) and UPEC-infected (C and E) cells. M; molecular weight marker, CM; cytokine mixture, UPEC; uropathogenic *E. coli*, NS; non-stimulated.

density at 450 nm in a Labssystem multiscan® plus fluorescence spectrophotometer.

2.7 Statistical analysis

Data are shown as mean $\pm$ standard error of the mean (SEM) and *n* indicates the number of independent experiments. Student's unpaired t-test or ANOVA, followed by Dunnett's post test, was used with statistical significance considered at $p < 0.05$.

3. Results

3.1 Adenosine receptor expression in UROtsa cells

RT-PCR analysis was used to examine the expression of adenosine receptor mRNA in

UROtsa cells. UROtsa cells were found to have a basal mRNA expression of the adenosine A_1 , A_{2A} and A_{2B} receptor subtypes, but transcript for the adenosine A_3 receptor could not be detected (figure 1a). The adenosine A_3 receptor primers used have previously been confirmed to detect human adenosine A_3 receptor transcript in neutrophils (Save et al., 2009). The expression of the adenosine A_{2B} receptor was most pronounced. Stimulation with a proinflammatory cytokine mixture (6 and 12 hours) (figure 1b) and UPEC strain IA2 (12 and 24 hours) (figure 1c) caused a small but consistent increase in adenosine A_{2A} receptor expression. By contrast, the expression of the adenosine A_{2B} receptor was unaltered after the same treatment (figure 1d and e).

Fig. 2. Effect of the adenosine A_{2A} receptor agonist CGS 21680 and the adenosine A_2 receptor CPCA on cAMP accumulation in non-pretreated (A), cytokine-pretreated (B) and UPEC-pretreated (C) UROtsa cells. The cAMP response to forskolin is also shown. Figure D shows cAMP production in UROtsa cells infected with IA2 for one hour. Results are given as pmol/ml and expressed as mean $\pm$ SEM ($n = 2-6$). Statistical significance vs un-stimulated cells, * $p < 0.05$ and *** $p < 0.001$

3.2 Effect of adenosine A_2 receptor activation on cAMP accumulation

The effects of the adenosine A_2 receptor agonist CPCA and the selective adenosine A_{2A} receptor agonist CGS 21680 on cAMP accumulation was investigated in non-pretreated (figure 2a), cytokine-pretreated (figure 2b) and UPEC-pretreated (figure 2c) cells. CPCA increased cAMP levels in UROtsa cells and under normal (non-pretreated) conditions the increase elicited by CPCA was significant at both 1 ($p < 0.05$) and 10 μ M ($p < 0.001$). The CPCA-mediated cAMP accumulation in cytokine-pretreated and UPEC-pretreated cells was significant at 10 μ M ($p < 0.05$ and $p < 0.001$, respectively). No significant changes in cAMP accumulation were found in response to CGS 21680 (0.1 and 1 μ M). Forskolin (10 μ M), a known activator of adenylyl cyclase, increased cAMP

accumulation to approximately the same level as CPCA (10 μ M) (figure 2). The intracellular cAMP levels in UROtsa cells infected with UPEC-strain IA2 for one hour was slightly, although not significantly, higher than in non-stimulated control cells (figure 2d).

3.3 Effect of adenosine A_2 receptor activation on intracellular phosphoproteins

To further investigate intracellular pathways involved in adenosine A_2 receptor activation in UROtsa cells we studied changes of phosphorylated proteins by a multiplex immunoassay. This assay simultaneously measures changes in eight phosphorylated proteins in the same sample. For validation, lysates of heat shocked/arsenite-, TNF- α - and EGF-stimulated HeLa cells were used as positive controls. Stimulation of UROtsa cells with EGF (50 ng/ml), known to activate many

Fig. 3. Analysis of intracellular phosphoproteins using a multiplex immunoassay after stimulation of UROtsa cells with the positive control EGF (50 ng/ml) for 7 minutes (A). The CREB response evoked by the adenosine A_2 receptor agonist CPCA (B) and the adenosine A_{2A} receptor agonist CGS 21680 (C) after 7 and 15 minutes of stimulation. Analysis of ERK 1/2 (D), STAT3 (E) and IκBα (F) are shown in response to CPCA and CGS 21680 after 7 minutes of stimulation. Results are given as mean fluorescence intensity (MFI) and expressed as mean $\pm$ SEM ($n = 4$). Statistical significance vs non-stimulated cells, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, NS; non-stimulated.

Fig. 4. IL-8 production in UROtsa cells. Panel A shows a representative figure of IL-8 production in UROtsa cells exposed to UPEC strain IA2 (10^6 , 10^7 and 10^8 CFU/ml) for 6 and 24 hours. Effect of the adenosine A_{2A} receptor agonist, CGS 21680 (B) and the adenosine A_2 agonist CPCA and the adenosine A_{2B} receptor agonist combination CPCA/SCH 58261 (C) on IL-8 production evoked by IA2 (10^8 CFU/ml) for 24 hours. The IL-8 production evoked by IA2 was set to 100%. (D) The effect of forskolin on IL-8 production. Data are presented as mean $\pm$ SEM (n = 3-5). Statistical significant vs non-stimulated cells, * p <0.05, NS; non-stimulated.

of the pathways included in the immunoassay, was used as an internal positive control. The response of the different phosphoproteins to EGF is shown in figure 3a. A strong induction of CREB was seen in CPCA-stimulated cells. After stimulation with CPCA 1 μ M and 10 μ M for 7 minutes CREB increased $\sim$ 10-fold (p <0.01) and $\sim$ 14 fold (p <0.001), respectively compared to non-stimulated control. The CPCA-induced increase of CREB after 15 minutes was $\sim$ 16 and 19-fold (p <0.001) at 1 and 10 μ M, respectively (figure 3b). The adenosine A_{2A} agonist CGS 21680 (0.1 μ M and 1 μ M) induced a small but significant (p <0.05) increase of CREB after 15 minutes (figure 3c). A significant decrease of ERK 1/2 (p <0.01) (figure 3d) and STAT3 (p <0.05) (figure 3e)

was observed in response to 1 μ M CGS 21680. By contrast, CPCA did not change ERK 1/2 or STAT3 (figures 3d, e). The levels of phosphorylated I κ B α , part of the NF- κ B-complex, appeared lower (non-significantly) in both CGS 21680 and CPCA-stimulated cells (figure 3f). Furthermore, CGS 21680 (1 μ M), but not CPCA, caused non-significant decreases in JNK and p38 (data not shown).

3.4 The effect of adenosine A_2 receptor activation on IL-8 secretion

Time-course experiments with different bacterial concentrations (10^6 , 10^7 , 10^8 CFU/ml at 6 and 24 hours) were first performed to obtain optimal conditions for studies of UPEC-evoked IL-8 production. A minor increase in IL-8 production was seen

with 10^8 CFU/ml after 6 hours, but the strongest IL-8 response was found after 24 hours of infection with 10^8 CFU/ml (figure 4a). Stimulation of cells with IA2 (10^8 CFU/ml) for 24 hours was therefore chosen to study the effect of adenosine A_2 receptor activation on IL-8 secretion after UPEC infection. The adenosine A_{2A} receptor agonist CGS 21680 (0.1 and 1 μ M) had no effect on basal or UPEC-evoked IL-8 production (figure 4b). The adenosine A_2 receptor agonist CPCA (1 and 10 μ M) or the “adenosine A_{2B} receptor agonist combination” CPCA + the adenosine A_{2A} receptor antagonist SCH 58261 (10 nM) did not affect basal or UPEC-evoked IL-8 production (figure 4c). Forskolin (10 μ M) significantly increased IL-8 production by $24 \pm 5\%$ ($n=5$) ($p<0.05$) (figure 4d).

4. Discussion

In the present study a basal expression of adenosine A_1 , A_{2A} and A_{2B} , but not A_3 , receptors was shown in the human uroepithelial cell line UROtsa. This is in line with previous studies conducted on T24 human bladder carcinoma cells (Phelps et al., 2006) and A498 human kidney carcinoma cells (Save et al., 2009). To evaluate whether the expression of adenosine A_{2A} and A_{2B} receptor mRNA is altered in UTI-like conditions, UROtsa cells were stimulated with UPEC or a mixture of pro-inflammatory cytokines, known to be present in the urine of patients with UTI (Sadeghi et al., 2005). A small but consistent up-regulation of the adenosine A_{2A} receptor was seen in response to both UPEC and pro-inflammatory cytokines. These results are supported by our previous findings that adenosine A_{2A} receptor expression is up-regulated in human urinary tract epithelial cells in response to UPEC and cytokines and in mouse bladder uroepithelium infected with UPEC (Save et al., 2009). The basal adenosine A_{2B} mRNA receptor expression was pronounced in UROtsa cells but appeared to be unaltered after UPEC- and cytokine-stimulation.

Next, we investigated whether the increased adenosine A_{2A} mRNA receptor expression in response to UPEC and cytokines was accompanied by an increased adenosine A_{2A} receptor signalling. Both high-affinity

adenosine A_{2A} and low-affinity adenosine A_{2B} receptors are coupled to stimulatory G_s proteins which lead to the production of cAMP (Fredholm et al., 2000). However, in our study the selective adenosine A_{2A} receptor agonist CGS 21680 failed to stimulate cAMP production in non-pretreated UROtsa cells as well as in cells pre-treated with UPEC or cytokines. These results suggest that the enhanced adenosine A_{2A} mRNA expression found in cells pre-treated with UPEC or cytokines was not correlated with an increased amount of functional receptors and/or enhanced receptor signalling. In agreement with our data, Phelps and co-workers (Phelps et al., 2006) did not detect cAMP elevation upon adenosine A_{2A} receptor activation in T24 bladder cells and neither was any increase in intracellular Ca^{2+} observed. These data may suggest that the adenosine A_{2A} receptor in human uroepithelial cells is functionally inactive or that cAMP- and Ca^{2+} -independent signalling pathways are used for activation. Calcium- and cAMP-independent signalling have been described for adenosine A_{2A} receptors in PC12 cell, where a Ca^{2+} -independent isoform of PKC phosphorylates and inhibits adenylyl cyclase isozymes and cAMP signalling (Lai et al., 1997).

Due to the lack of specific adenosine A_{2B} receptor agonists we used the general adenosine A_2 receptor agonist CPCA to stimulate adenosine A_{2B} receptors. Since the adenosine A_{2A} response was modest CPCA could be considered as an adenosine A_{2B} receptor agonist in this study. Activation of adenosine A_{2B} receptors caused a strong cAMP response similar to that of the adenylyl cyclase activator, forskolin. Pre-treating the cells with cytokines prior to stimulation with CPCA had no effects on the cAMP response, but the cAMP levels were overall lower in UPEC-infected cells. One explanation could be that the cells are affected by the presence of UPEC and that the viability of the cells is decreased. Another possibility may be that a desensitization of the cAMP-pathway has occurred during the UPEC-infection. It has recently been shown that UPEC stimulate a rise in cAMP in the human bladder epithelial cell line 5637 (Song et al., 2007a; Song et al., 2009), and a minor increase of cAMP was also confirmed in UPEC-infected UROtsa cells.

Since suppression of both CPCA- and forskolin-evoked cAMP accumulation was observed, the reduced CPCA signalling after UPEC pre-treatment is not adenosine A_{2B} receptor specific but most likely caused at the level of adenyl cyclase or down-stream targets.

To investigate pathways, other than cAMP, that could be involved in adenosine A₂ receptor activation in UROtsa cells we studied changes of several phosphorylated signalling proteins. In agreement with the cAMP-assay, activation of the adenosine A_{2B} receptor induced a strong induction of the cAMP-responsive elements binding protein CREB. In addition, adenosine A_{2A} receptor activation induced a small but significant increase of phosphorylated CREB. These data may suggest that adenosine A_{2A} receptor activation, after all, stimulates cAMP but below the detection limit of the EIA assay. Furthermore, we found a significant decrease of STAT3, a down-stream target of many inflammatory cytokines such as IL-6 (Hodge et al., 2005) and the MAPK ERK 1/2 by adenosine A_{2A}, but not A_{2B}, receptor stimulation. In addition, a small decrease in phosphorylation of the MAPKs JNK and p38 was found in response to adenosine A_{2A} receptor activation. It is generally believed that inhibition of pro-inflammatory mediators by adenosine A_{2A} receptor activation is mediated through increased cAMP-CREB activation and subsequently inhibition of NF-κB activity (Bshesh et al., 2002; Lukashev et al., 2004). The key event in NF-κB activation is IκBα phosphorylation (Tak and Firestein, 2001). In our study, both adenosine A_{2A} and A_{2B} receptor activation slightly decreased IκBα phosphorylation, indicating that adenosine activation through adenosine A_{2A} receptors and A_{2B} receptors may influence inflammation through inhibition of NF-κB. Taken together, a marked difference was observed between adenosine A_{2A} and A_{2B} receptor signalling in uroepithelial cells. A cAMP-CREB signalling pathway was found for the adenosine A_{2B} receptor, while adenosine A_{2A} receptor activation inhibited STAT3 and MAPK-signalling but failed to activate cAMP.

The anti-inflammatory characteristics of adenosine A₂ receptors involve inhibition of cytokine production (Bouma et al., 1994;

Linden, 2006; Zidek, 1999). While the adenosine A_{2A} receptor subtype is well established as anti-inflammatory there are some controversies whether the adenosine A_{2B} receptor subtype is pro- or anti-inflammatory (Linden, 2006). Studies in adenosine A_{2B} knockout mice have suggested that activation of adenosine A_{2B} receptors on certain cells, particularly macrophages, inhibits inflammation (Yang et al., 2006) while another study favour a pro-inflammatory profile for this receptor (Ryzhov et al., 2008). Because the pro-inflammatory cytokine IL-8 is essential for neutrophil transmigration across the infected urothelium (Svanborg et al., 2006), we examined the possible regulatory effect of adenosine A₂ receptors on IL-8 production. The results demonstrated that adenosine A_{2A} and A_{2B} receptor activation did not affect basal or UPEC-induced IL-8 release. Activation of adenyl cyclase by forskolin and adenosine A_{2B} receptors by CPCA increase cAMP levels to approximately the same extent but both had modest effects on IL-8 secretion. A minor increase of cAMP was seen in UPEC-infected cells while the production of IL-8 was prominent. Therefore, UPEC-evoked IL-8 production seems to be mediated by pathways other than from cAMP signalling, such as the NF-κB pathway. A recent study provided evidence that UPEC-evoked IL-6 release in the human bladder epithelial cell line 5637 involved both the classical NF-κB pathway but also a cAMP-CREB pathway (Song et al., 2007b). The cAMP-pathway resulted in an early IL-6 response but the amount of IL-6 generated by this pathway was less than for the NF-κB-associated pathway.

It has previously been found that the UPEC-evoked IL-6 response required NF-κB and p38 MAPK activation in T24 bladder carcinoma cells (Schilling et al., 2003) and serine/threonine kinases in A498 kidney cells (Hedlund et al., 1996). The induction of IL-8 by type 1 fimbriated UPEC involves the MAPK-family (p38, JNK and ERK) and activator protein-1 (AP-1) (Tsai et al., 2009). Phosphorylation of p38 is required for P-fimbriated UPEC activation of both AP-1 and NF-κB and the subsequently induction of IL-8 (Tsai et al., 2009). The UPEC strain used in this study express both type 1 and P fimbriae

and our preliminary data demonstrate up-regulation of p38, JNK, ERK1/2 and I κ B- α in UPEC-infected UROtsa cells (unpublished observations). Our data suggest that adenosine A_{2A} receptor activation inhibits many of the signalling pathways that are induced in host urinary tract epithelial cells by UPEC-infection. However, despite this adenosine A_{2A} receptor activation did not functionally suppress UPEC-evoked IL-8 production in UROtsa cells. Stimulation of adenosine A_{2A} receptors with CGS 21680 decreased TNF- α and macrophage inflammatory peptides, but not IL-8, in LPS-stimulated human neutrophils (McColl et al., 2006). Thus, it is possible that an anti-inflammatory profile of the adenosine A_{2A} receptor had been noted if other markers for inflammation than IL-8 had been evaluated. It is well accepted that adenosine A_{2A} receptor activation inhibits pro-inflammatory mediators in immune cells (Ohta and Sitkovsky, 2001), while immunomodulatory consequences of adenosine A_{2A} receptor activation in non-immune cells like epithelial cells requires more studies. We have previously shown that adenosine A_{2A} receptor activation reduces UPEC-evoked IL-6 production in a kidney epithelial cell line (Save et al., 2009), while a study performed on T24 bladder cells showed increased IL-8 production after adenosine A_{2B} receptor activation (Phelps et al., 2006).

In conclusion, the adenosine A_{2B} receptor is highly expressed in the uroepithelium and signals through the cAMP-CREB pathway. No evidence for a role of the adenosine A_{2B} receptor in regulation of the host uroepithelial response was found. The low expression adenosine A_{2A} receptor showed an increased mRNA expression during UTI-like conditions but the post-translational functional importance of this increase is uncertain. Activation of adenosine A_{2A} receptors caused inhibition of MAPK- and STAT3-signalling, but it did not functionally affect UPEC-evoked IL-8 production.

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