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The Effect of A_{2A} Adenosine Receptor Activation on C-C Chemokine Receptor 7 Expression in Human THP1 Macrophages During Inflammation

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Abstract

C-C chemokine receptor 7 (CCR7) and its chemoattractant agonist CCL21 promote cell migration and expression of pro-inflammatory proteins in an atherogenic environment. Since A_{2A} adenosine receptor activation reduces migration and inflammatory effects, we examined its effect on CCR7 expression and migration. CCR7 protein expression decreased by about a third in macrophages treated with A_{2A} receptor agonist CGS 21680 ($p=0.028$, $n=7$) and was reversed with antagonist, although mRNA levels increased twofold ($p=0.001$, $n=3$). Furthermore, macrophages treated with CGS 21680 showed a significant decrease in migration ($p=0.0311$, $n=7$). These results suggest that A_{2A} adenosine receptor activation not only modulates CCR7 expression in both normal and inflammatory environments but also regulates macrophage migration to CCR7-specific chemoattractants.

Keywords

A_{2A} adenosine receptor; macrophage; chemokine receptor migration

INTRODUCTION

Atherogenesis occurs when arterial injury leads to a persistent inflammatory response— inflammatory cells respond and migrate to the area, macrophages take up oxidized low-density lipoprotein and, in the process, become foam cells, undergo apoptosis, and accumulate around the lesion, forming a growing plaque that undergoes ineffective repair and may calcify, becoming a complex plaque [1–4]. Chemokines and their receptors play a role in atherogenesis and arterial repair by regulating the recruitment and function of pro-inflammatory and plaque-destabilizing cells [5–7].

The purine adenosine accumulates during tissue injury or stress and, like chemokines, can modulate immune response and cellular function through its receptor subtype A_{2A} [8–11]. A_{2A} adenosine receptor activation suppresses inflammation by regulating cytokine production and prevents atherogenesis by suppressing cell migration and reducing foam cell formation through reverse cholesterol transport [12–17]. The A_{2A} adenosine receptor also modulates chemokine receptor levels; in particular, it downregulates C-C chemokine receptor subtype 7 (CCR7) expression and function in T and dendritic cells [18–20]. The attraction of monocytes and macrophages to CCR7's agonists could be used to prevent atherogenesis—if fewer new macrophages migrate into the atherosclerotic plaque, further arterial injury may be prevented.

We hypothesized that A_{2A} adenosine receptor activation modulates CCR7 macrophage expression and migration. We found that while A_{2A} adenosine receptor stimulation increased CCR7 mRNA, it decreased protein expression in both noninflammatory and inflammatory environments. As a result, chemotaxis of cells to CCR7 agonists decreased. We also examined signaling at A_{2A} receptors for regulation of CCR7 receptors and found that, as with other functions, adenosine A_{2A} receptors regulate CCR7 expression by a pathway dependent on PKA and MAPK.

MATERIALS AND METHODS

Materials

The selective A_{2A} receptor agonist CGS21680 and its antagonist ZM 241385 were purchased from Tocris Cookson (Bristol, UK). The selective CCR7 agonists CCL19 and CCL21 and the cytokine IFN γ were obtained from R&D Systems (Minneapolis, MN). Epac agonist 8-pcPT-2'-O-Me-cAMP was purchased from Axxora Diagnostics (San Diego, CA) and PKA inhibitor PKI, corresponding to amino acids 5 through 24, from Promega (Madison, WI). Rac inhibitor 553502 and p38 inhibitor SB202190 were obtained from Calbiochem (San Diego, CA), and Mek inhibitor U0126 from Sigma-Aldrich (St. Louis, MO).

Cell Culture

Human monocytic THP1 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI-1640 media supplemented with 10% FBS, 1% penicillin and streptomycin, and 1% L-glutamine (Invitrogen, Carlsbad, CA) at 37°C, 5% CO₂. Cells were differentiated to macrophages by incubation with 25 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 48 h.

RNA Isolation and Real-Time RT-PCR

THP1 monocytes were differentiated to macrophages (700,000 cells per milliliter), given fresh media, and treated with combinations of CGS21680 (1 μ M and 100 nM) and ZM241385 (10 μ M) for 4 h. For inflammatory experiments, cells were pretreated with 500 U/mL IFN γ for 12 to 16 h. For signaling experiments, cells were pretreated for 1 h with PKA inhibitor (10 μ M), p38 inhibitor (10 μ M), Rac inhibitor (10 μ M), or Mek inhibitor (10 μ M). Media were then removed, and RNA was extracted using Trizol (Invitrogen) according

to the manufacturer's instructions. Of total RNA, 1.5 µg was used from each sample for first-strand, complementary deoxyribonucleic acid synthesis with random hexamer primers using an RNA PCR Core Kit (Applied Biosystems, Foster City, CA).

For quantification by real-time PCR, the following primer pairs were used: CCR7 F: 5'-TGACATGCACTCAGCTCTTG-3' R: 5'-AGGTTTTTCAGTCCCTGTGAC-3' (60°C, 136 bp). GAPDH F: 5'-AACATCATCCCTGCCTCTAC-3' R: 5'-CCCTCTTGCTGATGCCAAAT-3' (58°C, 358 bp). PCR product was detected using SYBR Green and the MxPro 3005P from Agilent Technologies (Cedar Creek, TX). Gene expression was quantified to a standard curve and normalized to GAPDH.

Western Blot Analysis

Monocytes were differentiated to macrophages (1.5 million cells per milliliter), given fresh media, and treated with the appropriate cytokines, agonists, and inhibitors for 48 h. Cells were then lysed using radio-immunoprecipitation assay buffer with protease inhibitor cocktail (Sigma-Aldrich) for 30 min at 4°C, centrifuged at 10,000 rpm for 10 min, and the supernatant recovered. Of protein, 35 µg was separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes (BioRad, Hercules, CA). Nonspecific antibody binding was blocked with Superblock T20 (Thermo, Rockford, IL) for 4 h at room temperature. The membrane was incubated with a primary antibody against CCR7 (goat polyclonal from Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4°C and then incubated with anti-goat secondary antibody conjugated to alkaline phosphatase (Santa Cruz Biotechnologies) for 1 h at room temperature, after washing with TTBS. Protein bands were visualized using electrochemifluorescence substrate and a Storm Phosphorimager (GE Healthcare). Then, the membrane was stripped and incubated with a primary antibody against beta-actin (mouse monoclonal from Abcam, Cambridge, MA) to be used as an internal, normalizing standard. Band intensity was quantified using Kodak Molecular Imaging software (Eastman Kodak, Rochester, NY).

In Vitro Chemotaxis Cell Migration Assay

Monocytes were differentiated into macrophages, given fresh media, and pretreated with the appropriate cytokines, agonists, and antagonists for 48 h. Two hundred thousand cells were then gently lifted using rubber scrapers and Dispase (Roche, Mannheim, Germany) and added to 5-µm chamber inserts cells from the QCM Cell Migration Assay Kit (Chemicon, Temecula, CA) with fresh media containing 1% FBS, 1% penicillin-streptomycin, and 1% L-glutamine in RPMI-1640 media (Invitrogen); 300 ng/mL CCL19 or CCL21 was added to starved media in the lower chamber as a chemoattractant. Cells were then incubated for 24 h, and contents of the lower chamber were detached, stained with fluorescein, and lysed according to the manufacturer's instructions. Migration was quantified using the Victor³ Plate Reader (Perkin Elmer).

Statistics

Data are reported as the mean±SEM. Differences between treatment groups were analyzed with Graph-Pad Prism (GraphPad Software, San Diego, CA) using one-way analysis of

variance followed by Tukey posttest, paired and unpaired Student's *t* tests. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

A_{2A} Adenosine Receptor Agonist CGS21680 Increases CCR7 mRNA Expression

There was a dose-dependent increase in CCR7 message in macrophages following treatment with the adenosine A_{2A} agonist CGS21680 ($p < 0.001$ vs. control, $n=3$), and co-treatment with the A_{2A} antagonist ZM241385 abrogated the increase $p < 0.01$ vs. CGS, $n=3$, Fig. 1a).

Cytokines, which are abundant in atherosclerotic lesions, may affect expression of such genes as A_{2A} adenosine receptor expression in THP1 cells. Indeed, IFN γ diminishes both expression and function of A_{2A} receptors and plays a central role in atherogenesis [13]. We therefore determined whether IFN γ altered A_{2A}-mediated increases in CCR7 mRNA. We were surprised to observe that, unlike other adenosine receptor functions, IFN γ did not alter adenosine A_{2A}-mediated increase in CCR7 message or the capacity of ZM241385 to reverse that effect ($p < 0.04$ vs. CGS, $n=3$, Fig. 1b).

A_{2A} Adenosine Receptor Agonist CGS21680 Decreases CCR7 Protein Expression

Because A_{2A} receptor stimulation increased mRNA for CCR7, we next determined whether A_{2A} adenosine receptor activation similarly regulated CCR7 protein expression. We were surprised to find that, in contrast to mRNA expression, CCR7 protein levels decreased by about a third ($p < 0.03$ vs. control, $n=7$), an effect that was reversed by the antagonist ($p < 0.05$ vs. CGS, $n=7$, Fig. 2a). Pretreatment with the cytokine IFN γ also decreased CCR7 protein levels with CGS21680 treatment ($p < 0.04$ vs. control, $n=3$) and reversed with ZM241385 ($p < 0.03$ vs. CGS, $n=3$, Fig. 2b).

CGS21680 Modulates CCR7-Mediated Migration in Normal and Pro-inflammatory Environments

Prior work has indicated that adenosine A_{2A} receptor stimulation retards chemotactic migration to CCR7 ligands by dendritic cells, and our studies indicate that adenosine A_{2A} receptor stimulation diminishes CCR7 expression on THP-1 cells [18]. We therefore determined whether adenosine A_{2A} receptor stimulation regulates chemotaxis to CCR7 ligands by THP-1 cells in the absence or presence of inflammation. CGS21680 pretreatment reduced migration by 17% to CCL21 ($p < 0.03$ vs. control, $n=7$) and by a similar amount after IFN γ pretreatment ($p < 0.03$ vs. IFN γ , $n=7$, Fig. 3). Migration to CCL19, a ligand for CCR7, was also reduced. As with the effects on CCR7 expression, the effects of the adenosine receptor agonist were reversed by the A_{2A} receptor antagonist ZM241385 ($p < 0.008$ vs. CGS, $n=7$, Fig. 4).

A_{2A} Adenosine Receptor-Induced Changes in CCR7 Expression Are Blocked with PKA, p38, and Mek Inhibitors

A_{2A} adenosine receptors stimulate adenylate cyclase activity and increase cellular cAMP content, which activates Epac and/or PKA, which activate signaling pathways including MAPK and p38 [21, 22]. To probe the signaling pathways involved, we used pharmacologic

inhibitors of protein kinase A and erk1/2 and p38 MAP kinases and observed their effect on the capacity of adenosine A_{2A} receptor stimulation to alter mRNA levels for CCR7. We found inhibitors of PKA and Mek, but not a p38MAPK inhibitor or an inhibitor of rac (Fig. 5a, b). In contrast, p38 and rac inhibition completely blocked the effect of A_{2A} receptor stimulation on CCR7 protein expression ($p<0.05$ vs. CGS with p38 inhibition, $n=4$, Fig. 5c, d).

DISCUSSION

Since A_{2A} adenosine receptor stimulation reduces inflammatory and atherogenic effects and CCR7 activation promotes these functions, we hypothesized that A_{2A} adenosine receptor activation could reduce CCR7 expression and thus modulate macrophage migration to inflamed sites or atherosclerotic plaques. Our results provide very interesting results regarding the effects of A_{2A} receptor ligation on CCR7 expression. We found that receptor ligation increases CCR7 mRNA by a PKA- and MEK-dependent pathway but diminishes surface expression of the protein by a p38MAPK-, rac-dependent pathway (Fig. 6). Clearly, the resulting reduction in CCR7 chemotactic function results from the diminished receptor expression/function.

Macrophages near or within an atherosclerotic plaque are exposed to inflammatory cytokines like IFN γ , which diminish A_{2A} adenosine receptor expression and function and might influence their ability to regulate CCR7 levels [13, 23]. Furthermore, reverse cholesterol transport experiments demonstrate that A_{2A} adenosine receptor function in macrophages is altered by IFN γ [12, 15, 17, 24]. Unlike other adenosine A_{2A} receptor-mediated effects, the A_{2A} receptor-stimulated effects on CCR7 mRNA and protein expression were unaffected by IFN γ .

The contrast between adenosine receptor-stimulated increases in CCR7 mRNA and decreases in CCR7 protein expression and function were surprising. One explanation for these observations is that chemokine receptor processing and transport via the endoplasmic reticulum and Golgi apparatus may be disrupted, degraded, or downgraded by adenosine A_{2A} receptor stimulation. Adenosine A_{2A} receptor stimulation may also increase the rate at which CCR7 receptors are cleared from the surface of the cell, as previously demonstrated for other chemotactic receptors in neutrophils [25–27]. Alternatively, translation of CCR7 message may be blocked by adenosine receptor-stimulated microRNAs; a database search has found some miRNAs with sequence similarity of more than 65% with CCR7, although they are not located in the 5' region considered most effective for translational blocking (mirbase.org) [28–31].

To determine a signal transduction pathway between A_{2A} adenosine receptor activation and CCR7 expression, we tested pharmacologic signal transduction inhibitors corresponding to previously demonstrated adenosine A_{2A} receptor signaling pathways. A_{2A} adenosine receptor stimulation can activate either the signaling proteins Epac or PKA [17, 32, 33]. PKA activation may then influence many possible downstream pathways, most commonly Erk with its upstream protein Mek and p38 with its upstream protein Rac [21, 34, 35]. We found PKA inhibition reversed A_{2A} adenosine receptor-induced changes in CCR7 message,

while Epac analog treatment failed to mimic expression, indicating a PKA-dependent pathway. Mek inhibition blocked change in CCR7 expression as well, while p38 and Rac inhibition did not affect CCR7 message but did change CCR7 protein expression. These results suggest A_{2A} adenosine receptor activation in THP1 macrophages leads to MAPK signaling through a PKA-dependent pathway for CCR7 expression.

Although we showed the presence of IFN γ did not hinder A_{2A} adenosine receptor's ability to reduce CCR7 expression and cell migration, the cytokines TNF α and interleukin-1 also affect A_{2A} adenosine receptor expression but were not examined [13]. A thorough examination of the other two cytokines with CGS21680, or a possible combination of all three, would give a better understanding of the receptor's function in an inflammatory, atherogenic environment.

An increasing body of literature demonstrates that adenosine and its receptors may play a role in the development and regression of atherosclerosis. Prior work demonstrates that adenosine A_{2A} receptor activation diminishes foam cell formation not only by increasing ABCA1 expression but by increasing its function as well [12, 36–39]. We now provide further evidence that adenosine and its receptors might play a beneficial role in atherosclerosis by inhibiting recruitment of macrophages to atherosclerotic plaques.

ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
CCL19	C-C chemokine ligand 19
CCL21	C-C chemokine ligand 21
CCR7	C-C chemokine receptor subtype 7
CGS21680	2-p-(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamido- adenosine
DMSO	Dimethyl sulfoxide
Epac	Guanine nucleotide exchange factor activated by cAMP
Erk	Extracellular signal regulated kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde phosphate dehydrogenase
IFNγ	Interferon gamma
MAPK	Mitogen-activated protein kinase
Mek	MAP kinase kinase
p38	p38 mitogen-activated protein kinase
PCR	Polymerase chain reaction
PKA	Protein kinase A
Rac	Subfamily of Rho GTPases

RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute media
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	Standard error mean
SYBR Green	<i>N,N'</i> -dimethyl- <i>N</i> -[4-[(<i>E</i>)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]- <i>N</i> -propylpropane-1,3-diamine
THP1	Human acute monocytic leukemia cell line
TNFα	Tumor necrosis factor alpha
TTBS	Tween/Tris-buffered saline ZM 241385–4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

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Fig. 1.

A_{2A} adenosine receptor agonist CGS21680 increases CCR7 mRNA expression and is reversed with antagonist ZM241385 in THP1 macrophages. Messenger RNA isolated from cells pretreated overnight in **a** regular media conditions or **b** IFN γ (500 U/mL) and then treated with combinations of CGS21680 (10^{-6} M and 10^{-7} M), ZM241385 (10^{-5} M), and DMSO as a negative control for 4 h and were subjected to real-time RT-PCR using specific primers. The expression level was quantified using real-time PCR and normalized to GAPDH. Data shown are the means $\pm$ SEM of the percentages of control from three

independent experiments. **a** vs. Control, * $p=0.001$; vs. CGS, # $p=0.01$; **b** vs. CGS, # $p=0.0382$.

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Fig. 2.

A_{2A} adenosine receptor agonist CGS21680 decreases CCR7 protein expression and is reversed with antagonist ZM241385 in THP1 macrophages. Cells pretreated overnight in **a** regular media conditions or **b** IFN γ (500 U/mL) and then treated with combinations of CGS21680 (10^{-6} M and 10^{-7} M), ZM241385 (10^{-5} M), and DMSO as a negative control for 48 h had 35 μ g of their protein lysate analyzed with SDS-PAGE and immunoblotting. **c** Representative immunoblots. The expression level was quantified using Kodak Image Analysis software and normalized to beta-actin. Data shown are the means $\pm$ SEM of the

percentages of control from **a** seven blots and **b** three blots. **a** vs. Control, * $p=0.028$; vs. CGS, # $p=0.0432$; **b** vs. Control, * $p=0.0386$, ** $p=0.0085$; vs. CGS, # $p=0.0234$.

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Fig. 3. CGS21680 treatment with pro-inflammatory cytokines decreases THP1 macrophage migration to CCR7 agonists. Cells were pretreated overnight with IFN_γ (500 U/mL) or $\text{TNF}\alpha$ (10 ng/mL) and then with CGS21680 (10^{-6} M) or DMSO as a negative control for 48 h. Cells were then lifted, added in suspension to 5- μ m upper chamber inserts, and migrated to a lower chamber with **a** CCL19 (300 ng/mL) or **b** CCL21 (300 ng/mL) for 24 h. Migration was quantified using fluorescein labeling of cells. Data shown are the means

$\pm$ SEM of the percentages of three independent experiments with CCL19 and six with CCL21. *vs.* Control, * $p=0.0311$, ** $p=0.0389$, *** $p=0.0196$. *vs.* IFN γ , # $p=0.0273$.

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Fig. 4.

CGS21680 treatment with and without IFN γ decreases THP1 macrophage migration to CCR7 agonists and is reversed with antagonist ZM241385. Cells were first pretreated overnight with IFN γ (500 U/mL) or TNF α (10 ng/mL) and then with combinations of CGS21680 (10^{-6} M), ZM241385 (10^{-5} M), or DMSO as a negative control for 48 h. Cells were then lifted, added in suspension to 5- μ m upper chamber inserts, and migrated to a lower chamber with CCL21 (300 ng/mL) for 24 h. Migration was quantified using fluorescein labeling of cells. Data shown are the means $\pm$ SEM of the percentages of six independent experiments—three with ZM. vs. Control, * $p=0.0311$, ** $p=0.0273$. vs. CGS, # $p=0.0078$, ## $p=0.125$.

Fig. 5.

A_2A adenosine receptor-induced increase of CCR7 mRNA expression and decrease of CCR7 protein expression in THP1 macrophages can be reversed with PKA, p38, and Mek inhibitors. Cells were treated with combinations of **a** 10^{-6} M or **b** 10^{-7} M CGS21680 with p38i (10 μ M), PKAi (10 μ M), Meki (10 μ M), Raci (10 μ M), Epac analog (10 μ M), and DMSO as a negative control for 4 h, and the messenger RNA collected and subjected to real-time RT-PCR using specific primers. The expression level was quantified using real-time PCR and normalized to GAPDH. Data shown are the means $\pm$ SEM of the percentages of control from three independent experiments. Cells were also treated with combinations of **c** 10^{-6} M or **d** 10^{-7} M CGS21680 and inhibitors for 48 h and had 35 μ g of their cell lysate analyzed with SDS-PAGE and immunoblotting. **e** Representative immunoblots. The

expression level was quantified using Kodak Image Analysis software and normalized to beta-actin. Data shown are the means $\pm$ SEM of the percentages of control from three to four blots. **a** vs. Control, * $p=0.065$, ** $p=0.0416$; **b** vs. Control, * $p=0.043$; **c** vs. Control, * $p=0.038$; vs. CGS, # $p=0.0504$; **d** vs. Control, * $p=0.0581$; vs. CGS, # $p=0.0204$.

Fig. 6.

The A_{2A} adenosine receptor uses signaling pathways downstream of PKA to regulate CCR7 expression. The A_{2A} adenosine receptor subtype's G-stimulatory protein subunit (G_s) activates adenylate cyclase (AC), which leads to production of cAMP and stimulation of primarily p38 signaling to decrease CCR7 protein and primarily MA-PK signaling to increase CCR7 mRNA in THP1 macrophages.