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RESEARCH PAPER

Stimulation of adenosine A_{2B} receptors induces interleukin-6 secretion in cardiac fibroblasts via the PKC-δ–P38 signalling pathway

Wei Feng, Yao Song, Chao Chen, Zhi Zhen Lu and Youyi Zhang

Institute of Vascular Medicine, Peking University Third Hospital and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Beijing, China

Background and purpose: Inflammatory response and cytokine activation are markedly stimulated after myocardial infarction, and contribute to cardiac remodelling. Interleukin-6 (IL-6), a pro-inflammatory cytokine, has pleiotropic effects on cardiac remodelling. Adenosine, released by all cell types, binds to a class of G protein-coupled receptors to induce various cardiovascular effects. The aim of this work was to investigate whether activation of adenosine receptors, particularly A_{2B} adenosine receptors, could stimulate IL-6 secretion in cardiac fibroblasts (CFs).
Experimental approach: ELISA was used to assess IL-6 concentration in supernatant, and immunostaining was used to analyse IL-6 protein level in CFs. The levels of phosphorylated and total p38, extracellular signal-regulated kinase, c-Jun N-terminal kinase and protein kinase C-δ (PKC-δ) were determined by Western blot analysis.
Key results: Adenosine-5′-*N*-ethyluronamide (NECA), a stable adenosine analogue, dose- and time-dependently stimulated IL-6 secretion in CFs. The effect of NECA was dose-dependently inhibited by an A_{2B} antagonist, and silencing of the A_{2B} receptor also inhibited IL-6 secretion. By using PKC isoform-selective inhibitors and translocation peptide inhibitors, the PKC-δ isoform was found to be involved in the up-regulation of IL-6 production. Inhibition of p38 by SB203580, and adenoviral transfer of dominant-negative p38 inhibited NECA-induced IL-6 production. Furthermore, PKC-δ functioned as an upstream regulator of p38 MAPK in this process.
Conclusions and implications: We demonstrated a novel relationship between adenosine and IL-6 secretion, in that IL-6 secretion induced by NECA was mediated by adenosine A_{2B} receptor activation in CFs and was dependent on a PKCδ–P38 pathway.
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Keywords: A_{2B} adenosine receptors; cardiac fibroblasts; IL-6; PKCδ

Abbreviations: 8-pCPT, 8-pCPT-2′-*O*-Me-cAMP; AR, adenosine receptor; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS21680, 2-(4-[2-carboxyethyl]-phenethylamino)adenosine-5′-*N*-ethyluronamide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ERK, extracellular signal-regulated kinase; IB-MECA, N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; IL-6, interleukin-6; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MRS1191, 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid,3-ethyl 5-(phenylmethyl) ester; MRS1754, 8-(4-[{(4-cyanophenyl)carbamoylmethyl}oxy]phenyl)-1,3-di(*n*-propyl) xanthine; NECA, adenosine-5′-*N*-ethyluronamide; Rp-cAMPS, Rp-adenosine-3′,5′-cyclic monophosphorothioate; SCH58261, 5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5c]pyrimidine

Introduction

Adenosine is a ubiquitous, endogenous nucleoside that modulates various physiological functions by interacting with its receptors on the cell surface. Four adenosine receptors have been identified (A_1, A_{2A}, A_{2B} and A_3), and so far, all belong to the G protein-coupled seven-transmembrane superfamily of cell surface receptors. A_1 and A_3 receptors negatively regulate adenylyl cyclase by coupling to $G_{i/o}$ protein, whereas A_{2A} and A_{2B} positively regulate adenylyl cylase by coupling to G_s proteins. The A_{2B} receptor also couples to G_q proteins (Fredholm *et al.*, 2001). Adenosine is produced by all cell types, and recognized as a major local (autocrine and paracrine)

Correspondence: Youyi Zhang, Institute of Vascular Medicine, Peking University Third Hospital, Beijing 100191, China. E-mail: zhangyy@bjmu.edu.cn
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regulator of tissue function, especially when energy supply acutely fails to meet cellular energy demand (Jacobson and Gao, 2006).

Within the cardiovascular system, adenosine plays important roles in various physiological processes (such as vasodilatation, negative chronotropy, dromotropy and inotropy), stimulation of angiogenesis, regulation of fibroblast growth and fibrosis, modulation of substrate metabolism and modulation of cardiovascular inflammatory responses. It also has a role in cardioprotection limiting oncotic and apoptotic forms of cell death and reversible injury (stunning) (Ashton *et al.*, 2007). Recently, evidence has been accumulated, suggesting that adenosine is an important regulator of the myocardial remodelling process in response to various stimuli. Plasma adenosine levels are elevated in experimental hypertension (Ohnishi *et al.*, 1988; Yamada *et al.*, 1992; 1995), and elevated plasma concentrations of the nucleoside have been observed in patients with congestive heart failure (CHF) regardless of cause (Funaya *et al.*, 1997).

Inflammatory cytokines are important factors for monitoring the progression of diseases in research and clinically. Elevated circulating, as well as intracardiac interleukin-6 (IL-6) levels, have been reported in patients with CHF, and high plasma levels of IL-6 can provide prognostic information in patients with CHF (MacGowan *et al.*, 1997; Roig *et al.*, 1998; Tsutamoto *et al.*, 1998). Hence, IL-6 might contribute to the progression of myocardial damage and dysfunction in chronic heart failure syndrome of different origins. Although the IL-6 family plays a crucial role in the pathophysiology of cardiovascular diseases, whether the IL-6 family is beneficial or detrimental remains to be determined (Kanda and Takahashi, 2004).

Increasing evidence suggests that adenosine signalling plays a role in the regulation of processes in the cytokine network. Adenosine increases the release of IL-6 from human lung fibroblasts, bronchial smooth muscle cells, astrocytes and pituitary folliculostellate cells (Schwaninger *et al.*, 1997; Rees *et al.*, 2003; Zhong *et al.*, 2004; 2005). The myocardium is believed to be a major source of IL-6 in patients with acute myocardial infarction and heart failure (Wan *et al.*, 1996). Our laboratory has previously reported that adult mouse cardiac fibroblasts (CFs), but not cardiomyocytes, are the predominant source of IL-6 in the mouse myocardium (Yin *et al.*, 2003). In the present study, we aimed to investigate whether adenosine receptors mediate the production of IL-6 in adult mouse CFs and the underlying mechanism. Here, we show that the stable adenosine analogue adenosine-5′-*N*-ethyluronamide (NECA) dose- and time-dependently stimulated IL-6 secretion in CFs, and that this effect is mediated by the adenosine A_{2B} receptor subtype. Furthermore, NECA-induced IL-6 release was independent of the Gs–cAMP–PKA pathway, but required protein kinase C-δ (PKC-δ), as well as p38 MAPK activation.

Methods

Cell culture

Adult mouse CFs were isolated from 6 to 8 week male Balb/c mice weighing 20–30 g obtained from the Medical Experimental Animal Centre of Peking University Health Science Centre. Minced ventricles were digested with 0.01% collagenase II, and the first 40 min digestion was discarded. The cells in the four following 15 min periods of digestion were collected, and fibroblasts were allowed to attach to 10 cm cell culture plates in 10 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 $U \cdot mL^{-1}$ penicillin/streptomycin and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) for 2 h at 37°C. Two hours later, the unattached cardiomyocytes were removed. After a rinse with DMEM twice, the CFs were cultured in DMEM with 10% FBS at 37°C for 4 days until they reached confluence.

Cells at the first passage were used in this experiment, and immunostaining and examination of morphology demonstrated that the cultured cells were pure CFs. The cultured cells exhibited positive staining for vimentin, and negative staining for von Willebrand factor, α-smooth muscle actin and α-sarcomeric actin, which indicates no relevant contamination of endothelial cells, smooth muscle cells or cardiac myocytes.

NIH3T3 cells were cultured in DMEM containing 10% FBS, and cells were seeded in six-well plates 1 day before transfection at a concentration of 1×10^5 cells per well. To each well, 1 μg of DNA was mixed with 3 μL of LipofectAMINE in 0.5 mL of Opti-MEM, and 6 h later, 0.5 mL of 20% FBS in DMEM was added to the medium. After 48 h, the cells were assayed for mRNA expression by the RT–PCR method.

ELISA

The concentration of IL-6 in the culture supernatant was measured by a commercially available ELISA kit according to the manufacturer's instructions. All samples were assayed in triplicate.

Immunofluorescence microscopy

The CFs were washed with phosphate-buffered saline (PBS) twice, and were fixed with 4% paraformaldehyde for 20 min, then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After the cells had been washed with PBS, non-specific binding sites were blocked with 1% normal goat serum in PBS for 60 min. The primary antibody was against IL-6 (diluted 1:200), and the secondary antibody was FITC-conjugated anti-rabbit IgG antibody (diluted 1:400). After a wash with PBS, the CFs were examined under fluorescent microscopy (DM IRB; Leica, Wetzlar, Germany).

RT–PCR

Total RNA was extracted by using TRIZol. One microgram of total RNA was then reverse transcribed into cDNA by use of the RT–PCR Access Kit. One microlitre of cDNA was used for PCR. Mouse $A_{2B}R$ and GAPDH mRNA were amplified by PCR with the following primers: mouse A_{2B}, 5′-AGCTAGAGA CGCAAGACGC-3′ and 5′-GTGGGGGTCTGTAATGCACT-3′; and mouse GAPDH, 5′-TGGCCTCCAAGGAGTAAGAA-3′ and 5′-GGTCTGGGATGGAAATTGTG-3′. The PCR mixture was incubated in a DNA Thermal Cycler (Stratagene, La Jolla, CA,

USA). After amplification, the products were analysed by electrophoresis on 10% polyacrylamide gel.

To determine the adenosine receptor subtypes expressed by CFs, RT–PCR was also performed. The primer pairs are the following oligonucleotides: Fw 5′-ATCCCCACCGCAGGCTT CCT-3′ and Rev 5′-AGGGCGCCAACAGCCACATC-3′ (A_1), Fw 5′-GCCATCCCATTCGCCATCA-3′ and Rev 5′-GCAATAGCCA AGAGGCTGAAGA-3′ (A_{2A}), Fw 5′- CCACCAACTACTTTCTGG TATCC-3′ and 5′-GTGTCCCAGTGACCAAACCT-3′ (A_{2B}), Fw 5′-CATGCTTCCATCATGTCCTTGC-3′ and 5′- GCTCGCTAAG GTTGCTTTTCT-3′ (A_3).

Constructs of mouse adenosine A_{2B} receptor small interfering RNA (siRNA)

For silencing the expression of A_{2B} receptors, three siRNAs targeting the different regions of mouse A_{2B} receptor gene were selected and constructed in the pAdTrack-HP vector (Zhao *et al*., 2003). The target sequences for mouse A_{2B} receptor (GenBank accession NM_007413) were 241–259 CAC CAACTACTTTCTGGTA; 1011–1029, GCTACAGTTTCCACA AGAT; and 652–670, CATGAGCTACATGGTGTAT, chemically synthesized as complementary oligonucleotides, annealed and then ligated into the BglII/HindIII sites of pAdTrack-HP. The recombinant shuttle vector was then co-transformed into *Escherichia coli* BJ5183 cells by use of a pAdEasy-1 adenoviral backbone plasmid (He *et al*., 1998). A recombinant virus was produced in human embryonic kidney (HEK) 293A cells, and purified by the ViraBind Adenovirus Purification Kit. Viral titre was determined by testing the multiplicity of infection in HEK 293A cells, and was used to infect cells at 50 plaque-forming units per cell. Under these conditions, approximately 98% of cells were infected, as assessed by infection with the same titre of Ad-GFP.

Adenovirus preparation and cell infection

Dominant-negative p38 recombinant adenovirus was purchased from Cell Biolabs (San Diego, CA, USA), and the adenoviruses were propagated in HEK 293 cells and purified by use of the ViraBind Adenovirus Purification Kit. CFs were infected at a multiplicity of infection of 50 plaque-forming units per cell in DMEM supplemented with 10% FBS for 6 h at 37°C and 5% CO_2, followed by the addition of a fresh medium containing FBS for an additional 18 h. The medium was replaced 24 h after infection with DMEM containing 10% FBS for an additional 24 h before treatment.

Cyclic AMP accumulation assay

Confluent cells in six-well plates were pre-labelled with [^{3}H]-adenine (1 μ Ci per well) for 4 h, and the medium was removed and the cells were washed twice with warm Krebs–Ringer bicarbonate (KRB) buffer (composition in mM:120, NaCl; 5.5, KCl; 2.5, $CaCl_2$; 1.2, NaH_2PO_4; 20, $NaHCO_3$; 11, glucose; and 0.029, $CaNa_2EGTA$, 37°C). Then, drugs were added in 1 mL of KRB containing 200 μM IBMX. Cells were incubated at 37°C for 10 min, and reactions were terminated by adding 100 μL of 77% trichloroacetic acid and 50 μL of 100 mM cAMP. Aliquots of 50 μL were counted for incorporation of [^{3}H]-adenine, and [^{3}H]-cAMP was isolated from the remaining supernatant by sequential Dowex-50 and alumina chromatography.

Peptide treatments

After being deprived of serum for 24 h, the cells were treated with NECA or different isozyme-selective PKC peptide inhibitors (myristoylated δV1-1 or myristoylated εV1-2) each at a final extracellular concentration of 5 mM. Cell viability and morphology were not affected by the myristoylated peptides. The ability of myristoylation of peptides to introduce peptides into cells has been described previously (Ward and O'Brian, 1993; Nelson *et al*., 2007).

Western blot analysis

CFs were initially grown to the desired confluency in growth media, and rendered quiescent by serum starvation for 24 h. After the cell samples were lysed in 80 μL lysis buffer, the protein concentration was estimated by the BCA protein assay kit. Proteins (30 μg) were loaded onto 10% SDS polyacrylamide gel, and electrophoretically transferred to nitrocellulose membranes (Pall, New York, NY, USA). The sheets were analysed with antibodies according to the supplier's protocol, and immunolabelled bands were visualized by use of the SuperSignal West Pico chemiluminescence kit.

Statistical analysis

Results are reported as mean ± SD with n representing the number of experiments on cells. Differences between normalized data were analysed by Student's unpaired t-test. Dose–response data were compared as ratios by use of repeat-measure one-way ANOVA and Newman–Keuls *post hoc* test. All statistical analyses involved the use of GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA, USA). A $P < 0.05$ was considered statistically significant.

Materials

Collagenase II was obtained from Worthington (Freehold, NJ, USA); LipofectAMINE and TRIZol from Invitrogen (Carlsbad, CA, USA); Opti-MEM from Life Technologies (Grand Island, NY, USA); the ELISA kit from R&D Systems (Minneapolis, MN, USA); the RT–PCR Access kit from Promega (Madison, WI, USA); the ViraBind Adenovirus Purification kit from Cell Biolabs (San Diego, CA, USA). The *E. coli* BJ5183 cells and the pAdEasy-1 adenoviral backbone plasmid were both kindly provided by Dr B. Vogelstein (Johns Hopkins University, Baltimore MD, USA). The BCA protein assay kit was obtained from Pierce (Rockford, IL, USA), while the SuperSignal West Pico chemiluminescence kit was from Perbio (Cramlington, Northumberland, UK).

Adenosine, 2-chloro-N^6-cyclopentyladenosine (CCPA), 2-(4-[2-carboxyethyl]-phenethylamino)adenosine-5′-N-ethyluronamide (CGS21680), NECA, N^6-(3-iodobenzyl) adenosine-5′-N-methyluronamide (IB-MECA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 5-amino-2-(2-furyl)-7-phenylethyl-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5c]pyrimidine

(SCH58261), 8-(4-[{(4-cyanophenyl)carbamoylmethyl}oxy] phenyl)-1,3-di(*n*-propyl)-xanthine (MRS1754), 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid,3-ethyl 5-(phenylmethyl) ester (MRS1191), Rp-Adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS), H-89, KT5720, 8-pCPT-2′-*O*-Me-cAMP (8-pCPT), Ro-31-8220, SP600125 and SB203580 were purchased from Sigma-Aldrich (St Louis, MO, USA). U-73122, U0126 and GÖ 6976 were purchased from Calbiochem (San Diego, CA, USA). The nomenclature used for the adenosine receptors, agonists and antagonists was as described by Alexander *et al*. (2008). Rabbit polyclonal antibodies against phosphorylated p38, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and PKC-δ were purchased from Cell Signaling Technology (Danvers, MA, USA), and rabbit polyclonal antibodies against p38, ERK, JNK, PKC-δ and IL-6 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). δV1-1([Myr]-SFNSYELGSL); amino acid 8–17 in PKCδ) and εV1-2([Myr]-EAVSLKPT; amino acid 14–21 in PKCε) were synthesized at the peptide facility at Shanghai Sangon Company. Myristoylated peptides were produced by incorporating a myristoylated lysine during solid phase synthesis. The peptides were >98% pure. The peptides were dissolved in deionized water, aliquoted and stored at –70°C.

Results

NECA stimulated IL-6 production in CFs

To assess whether stimulation of adenosine receptors could induce IL-6 release in CFs, we measured the effects of NECA, a non-selective adenosine receptor agonist, on IL-6 release by CFs by ELISA. The cells were incubated with NECA from 0.1 nM to 10 μM for 12 h, then the concentration of IL-6 was detected. NECA induced IL-6 secretion dose-dependently (Figure 1A). The EC_{50} for NECA was 154 ± 2.4 nM. The IL-6 concentration was significantly increased at 3 h as compared with the controls, and then further increased in a time-dependent manner in response to NECA ($P < 0.05$) (Figure 1B). IL-6 production in response to NECA stimulation was also confirmed by immunofluorescence staining (Figure 1C).

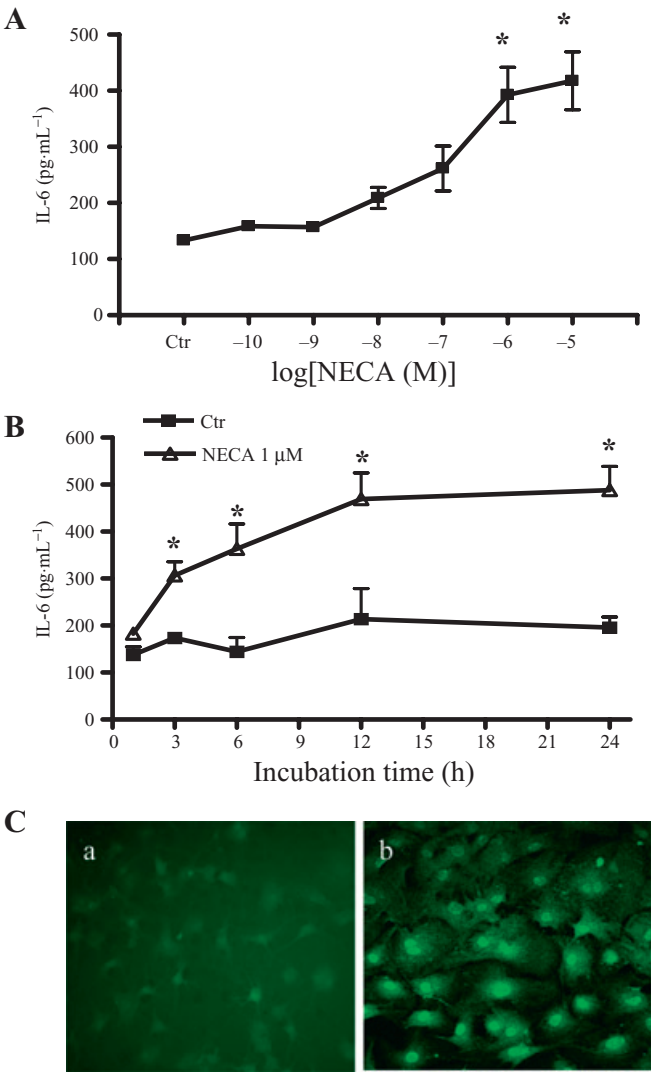

Figure 1 NECA stimulated IL-6 production in CFs. Concentration- and time-dependent effects of NECA on the IL-6 production in CFs. (A) CFs were treated with vehicle (Ctr) or the indicated concentrations NECA for 12 h and (B) vehicle (Ctr) or NECA (1 μM) for the indicated times. The supernatant concentration of IL-6 in CFs was assayed by ELISA. Values are means ± SD of four independent experiments. *$P < 0.05$ versus control. (C) Immunostaining of CFs stimulated with 1 μM NECA (b) for 6 h showed that the intensity of the staining for IL-6 was more in these cells than in the control cells (a).

A_{2B} receptor-mediated NECA-induced IL-6 secretion in CFs

To determine which subtype of adenosine receptor mediates the IL-6 release in CFs, the adenosine receptor subtype-selective agonist CCPA (subtype A_1 selective), CGS21680 (subtype A_{2A} selective) and IB-MECA (subtype A_3 selective) were used. The concentrations applied could selectively activate the indicated subtype. Only adenosine and NECA significantly induced IL-6 secretion in the supernatant; CCPA, CGS21680 and IB-MECA had no effect (Figure 2A). The effects of DPCPX, SCH58261, MRS1754 and MRS1191, the selective antagonists for A_1, A_{2A}, A_{2B} and A_3 adenosine receptors, respectively, were investigated. Only MRS1754, the A_{2B} subtype antagonist, dose-dependently inhibited NECA-induced IL-6 release. To further confirm the role of the A_{2B} receptor, three coding regions of the A_{2B} receptor gene were selected as targets for RNA interference to silence the A_{2B} receptor gene in CFs. Knockdown of the A_{2B} receptor by adenoviral transfection, and expression of short hairpin RNA significantly reduced the NECA-induced release of IL-6 (Figure 2C and D).

The G_s–cAMP–PKA signalling pathway is not involved in NECA-induced IL-6 release in CFs

The A_{2B} receptor has been found to couple to different signalling pathways, including the G_s–cAMP–PKA pathway and the G_q/G_{11}–PLC–PKC pathway (Feoktistov and Biaggioni, 1997). To investigate whether the G_s–cAMP–PKA pathway is involved in NECA-induced IL-6 expression in CFs, the CFs were pretreated with a cAMP competition analogue, Rp-cAMPS, and two PKA inhibitors, H-89 and KT5720, for 30 min before further treatment with 1 μM NECA for 6 h.

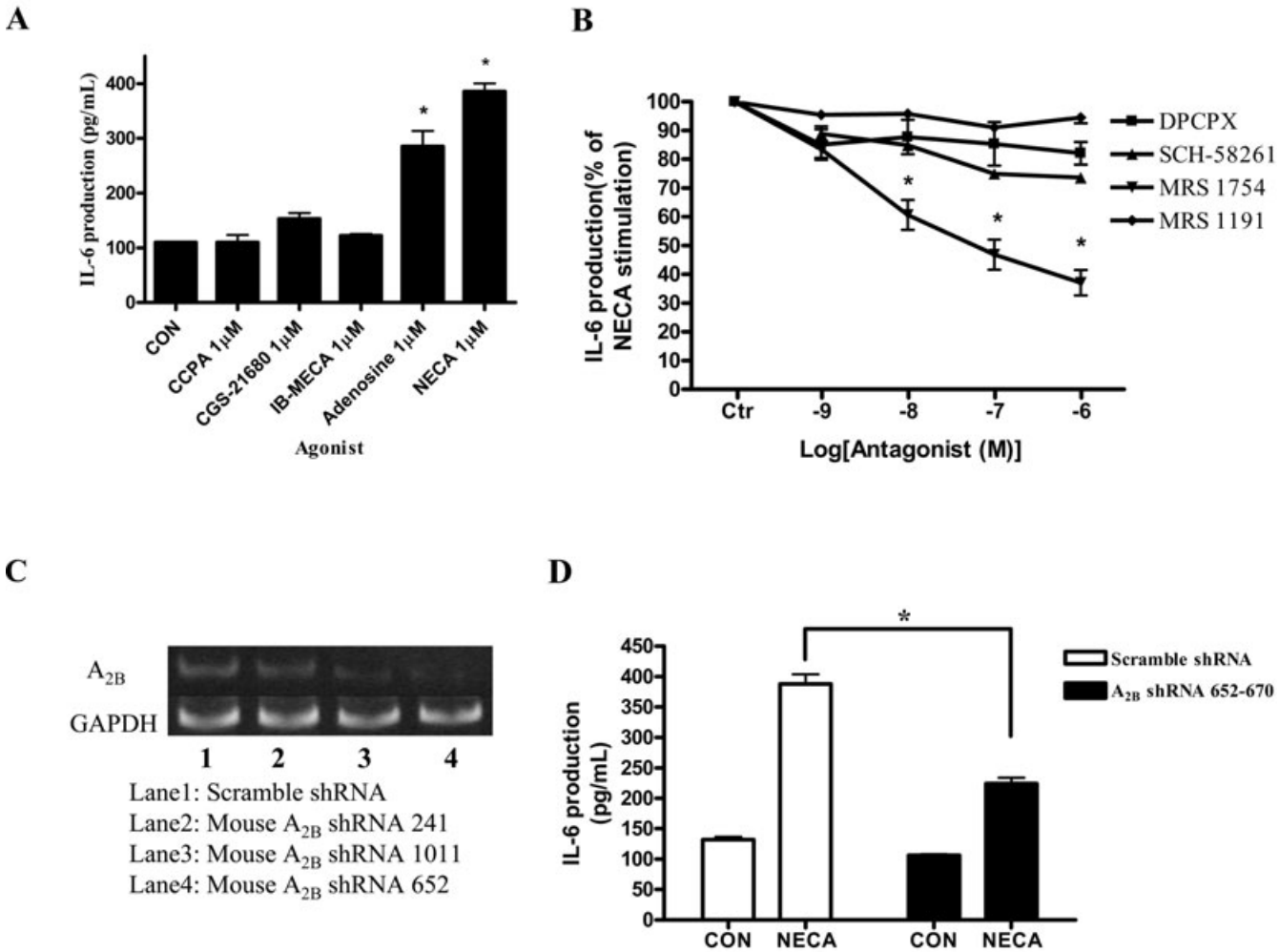

Figure 2 A_{2B} adenosine receptor stimulation-mediated IL-6 production in CFs. (A) CFs were incubated with control (DMSO) or different adenosine receptor agonists at the indicated concentrations for 6 h, and IL-6 concentrations in the supernatant were determined by ELISA. Data are mean ± SD presented as percentage increase above basal IL-6 production (100%). *$P < 0.05$ versus control. (B) CFs were pre-incubated for 30 min with control solution (DMSO) or the indicated concentrations of different adenosine receptor antagonists DPCPX, SCH28621, MRS1754, MRS1191 before being stimulated with 1 μM NECA for 6 h. Supernatants were harvested, and IL-6 protein secretion was quantified by use of an IL-6 ELISA kit. Data are mean ± SD given as percentages of the stimulation by NECA of IL-6 synthesis (100%) in the absence of antagonists. *$P < 0.05$ for various concentrations of antagonists on IL-6 production in response to 1 μM NECA alone. (C) RT–PCR analysis after transfecting the NIH 3T3 cells with the pAdTrack-HP vector targeting different regions of the A_{2B} receptor gene for 48 h. (D) The CFs were transfected with recombinant adenovirus carrying short hairpin RNA targeting the gene of mouse A_{2B} receptor 652–670 for 24 h, then deprived of serum for 24 h followed by 6 h of treatment with 1 μM NECA. The supernatants were harvested, and the expression of IL-6 was determined by ELISA. Data are expressed as mean ± SD values. *$P < 0.05$.

These inhibitors did not reduce the accumulation of IL-6 in the supernatant induced by NECA (Figure 3A). To investigate the role of Epac, another cAMP downstream effector, in the NECA-induced IL-6 release, we used a cAMP analogue, 8-pCPT-2′O-Me-cAMP to specifically activate Epac, and found that Epac activation did not induce the release of IL-6 (Figure 3B).

The G_q–PLC–PKC pathway participates in NECA-induced IL-6 release in CFs

PKC-specific inhibitors were used to elucidate the role of the G_q–PLC–PKC pathway in NECA-induced IL-6 release. The PLC_β inhibitor U73122 and PKC inhibitor Ro 31-8820 dose-dependently inhibited NECA-induced IL-6 release (Figure 4A). A more specific PKC inhibitor, GÖ 6976, a potent inhibitor of the activity of PKC-α, -βI and -μ at the nanomolar level, had no significant influence on NECA-induced IL-6 release up to a dose of 100 nM. In addition, inhibition of endogenous PKCδ with myristoylated δV1-1, an isozyme-selective translocation inhibitor, inhibited NECA-induced IL-6 release. However, inhibition of endogenous PKCε translocation with myristoylated εV1-2 had no effect on IL-6 release (Figure 4C). The phosphorylation of two sites for PKC activity by PKC-δ was further investigated by Western blot analysis. Thr505 phosphorylation was decreased in the first 60 min, but was markedly increased after 90 min upon NECA treatment, and Ser643 phosphorylation also showed a delayed pattern of increase (Figure 4D).

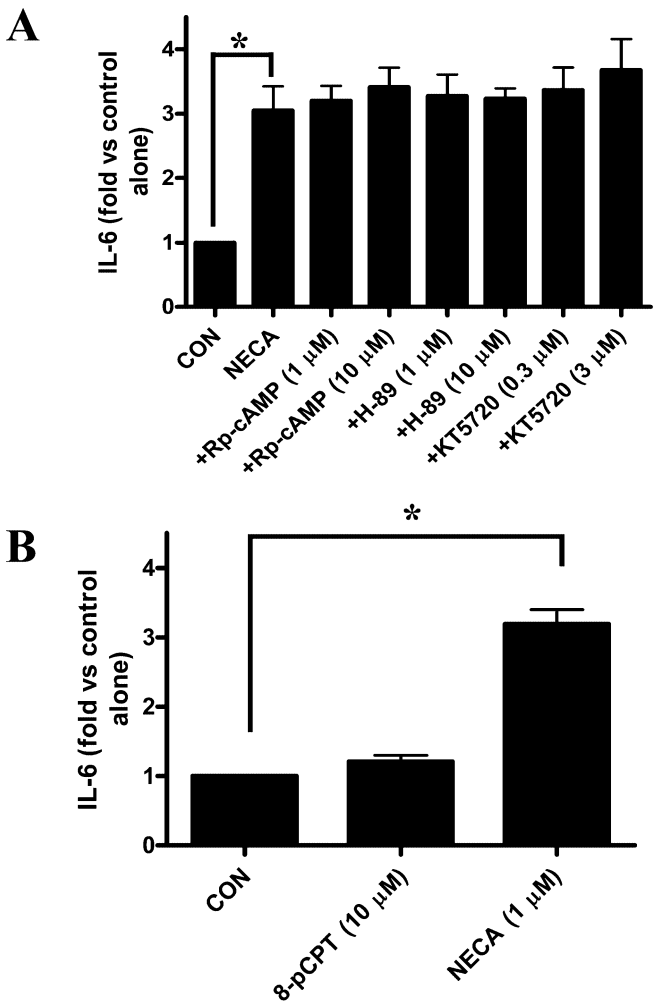

Figure 3 The Gs–cAMP–PKA pathway is not involved in NECA-induced IL-6 secretion. (A) The effect of Rp-cAMPS and two PKA inhibitors, H-89 and KT5720, on NECA-stimulated IL-6 secretion from CFs. Cells were treated with inhibitors at the indicated concentrations for 30 min before incubation with 1 μM NECA for 6 h. IL-6 was measured by ELISA. (B) Effect of the Epac agonist 8-pCPT on IL-6 secretion. CFs were treated with 10 μM 8-pCPT for 6 h, then IL-6 in the supernatant was measured. All results are represented as mean ± SD of three independent experiments. *$P < 0.05$.

P38, but not ERK and JNK MAPK, is involved in NECA-induced IL-6 release

The p38-specific inhibitor SB203580, ERK-specific inhibitor U0126 and JNK-specific inhibitor SP600125 were used to investigate the role of p38, ERK and JNK in NECA-induced IL-6 release in CFs. Cells were pre-incubated with each inhibitor for 30 min, and then stimulated for 6 h with 1 μM NECA. SB203580 substantially decreased IL-6 levels, as determined by ELISA, whereas U0126 and SP600125 had no effect on IL-6 levels (Figure 5A). We subsequently investigated, by Western blot analysis, the activation/phosphorylation status of the three MAPKs after NECA treatment. Phosphorylation of p38 MAPK peaked after 5 min of stimulation with 1 μM NECA and persisted for 90 min (Figure 5B), whereas that of ERK and JNK was not altered by NECA stimulation. In addition, we infected CFs with a recombinant adenovirus containing a dominant-negative mutant of p38 (Ad-DN-p38) to specifically inhibit p38 MAPK (Figure 5C); NECA-induced IL-6 release was markedly suppressed in these infected CFs (Figure 5D). In addition, p38 activation induced by NECA could be abrogated by the PKC-δ isoform translocation inhibitor peptide, which suggests that PKC-δ is the upstream signal of p38 MAPK (Figure 5E).

Discussion and conclusions

In the present study, we found that adenosine and its stable adenosine analogue NECA increased the expression and release of IL-6 by CFs, and this effect of NECA was mediated by the A_{2B} adenosine subtype. To our knowledge, this is the first time that adenosine has been shown, via this particular receptor subtype, to have an effect on inflammatory cytokine release by CFs, and may represent a novel mechanism for the role of adenosine in the cytokine network.

Adult mouse CFs express four subtypes of adenosine receptor (Supporting Information Figure S1). In this study, we obtained evidence that the A_{2B} adenosine receptor mediated the effects of the stable adenosine analogue NECA on IL-6 release. The non-selective adenosine agonist NECA increased the expression and release of IL-6, whereas CCPA, CGS21680 and IB-MECA, selective agonists for A_1, A_{2A} and A_3 adenosine receptors, respectively, had no effect on IL-6 release; these agonists are very potent at the cognate receptors without having significant activation of the A_{2B} adenosine receptors. In addition, the effects of NECA on IL-6 release were dose-dependently counteracted by MRS1754, a specific A_{2B} receptor antagonist, with the expected rank order of potency (K_i value 2 nM) (Kim *et al.*, 2000). Finally, knockdown of A_{2B} receptors in CFs, by an adenovirus expressing a short hairpin RNA that targets the adenosine A_{2B} receptor, abrogated the NECA-induced IL-6 secretion.

These results are in agreement with those obtained in various cell types of different origins, such as pituitary folliculostellate cells (Rees *et al.*, 2003), bronchial smooth muscle cells (Zhong *et al.*, 2004), osteoblasts (Evans *et al.*, 2006), astrocytoma cells (Fiebich *et al.*, 2005), astroglioma cells (Fiebich *et al.*, 1996) and astrocytes (Schwaninger *et al.*, 1997), that all show NECA-induced IL-6 release was via the A_{2B} receptor. Recently, Sergey *et al.*, using A_{2B} adenosine receptor knockout mice, indicated that genetic ablation of A_{2B} receptors abrogated NECA-induced IL-6 release from mouse peritoneal macrophages (Ryzhov *et al.*, 2008). Vazquez *et al.* (2008) also demonstrated that NECA evoked IL-6 release via activation of A_{2B} receptors *in vivo*. Accordingly, A_{2B} receptors have been suggested to mediate the pro-inflammatory actions of adenosine.

The failure of the Rp-cAMPS H-89 and KT5720 to inhibit NECA-induced IL-6 formation was unexpected, because it is well documented that the G_s protein-coupled A_{2B} receptor increases the formation of cAMP. Other researchers had demonstrated that cAMP elevation induced IL-6 release in various cells (Kiriyama *et al.*, 1997; Chio *et al.*, 2004; Carapau *et al.*, 2007). In addition, recently, it has been suggested that NECA increases cAMP concentration in rat CFs (Epperson *et al.*, 2009). However, we did not observe any increase in cAMP accumulation after NECA treatment for 10 min in the adult mouse CFs (Supporting Information Figure S2). These data

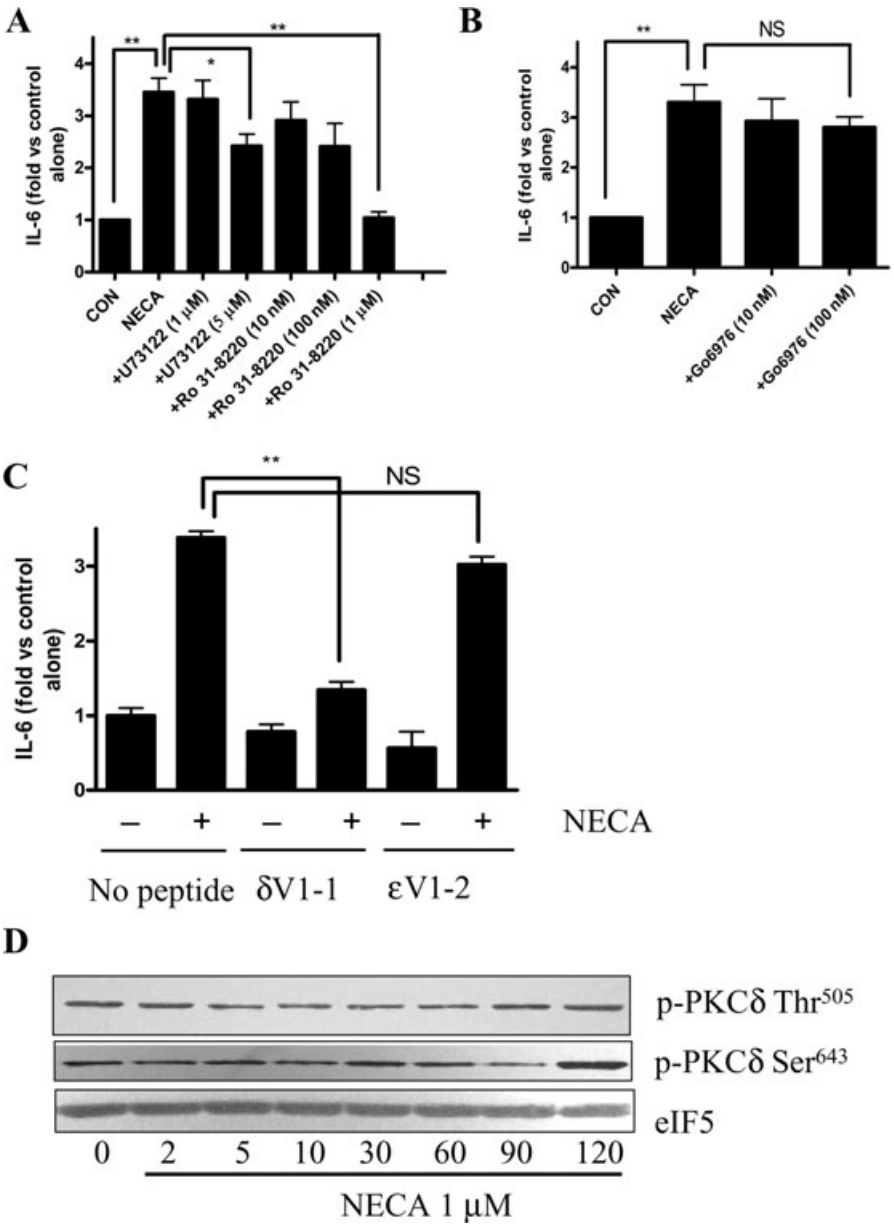

Figure 4 The Gq–PLC$_\beta$–PKC pathway is involved in NECA-induced IL-6 production in CFs. Cells were pre-incubated with the specific PLC$_\beta$ inhibitor U73122 or PKC inhibitor Ro 31-8220(A), or PKC isozyme-selective inhibitors GÖ 6976 (B) at the indicated concentrations for 30 min, and then stimulated with 1 μM NECA for 6 h. IL-6 in the supernatant was quantified by ELISA. (C) Cells were pre-incubated with the PKC-δ-selective inhibitor peptide (myristoylated δV1-1, δV1-1) or PKC-ε-selective inhibitor peptide (myristoylated εV1-2, εV1-2), or no peptide at the indicated concentrations for 60 min and then stimulated with 1 μM NECA for 6 h. IL-6 in the supernatant was quantified by ELISA. Data are expressed as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$. (D) Cells were treated with 1 μM NECA for 2–120 min, then the levels of PKC-δ phosphorylation at residues Thr^{505} and Ser^{643} were determined by Western blot analysis. All data are expressed as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$.

suggest that adenosine A_{2B} receptors mediate IL-6 release through a cAMP-independent mechanism in mouse CFs. Hence, whether or not cAMP is involved in the regulation of NECA-induced IL-6 synthesis might depend on cell type, and needs to be investigatedfurther.

Earlier findings showing that PKC inhibitors prevent NECA-induced IL-6 production have indicated that PKC has a role in the regulation of IL-6 formation (Rees *et al.*, 2003). It has been reported that CFs express six PKC isoforms (α, δ, ε, βI, βII, ζ) (Zhong *et al.*, 2004). Elucidation of the role of a particular PKC isoenzyme in a specific cellular response is complicated by the concomitant expression of several isoenzymes in a given cell type, as well as by the lack of isoenzyme-specific activators and inhibitors. In our present investigation, particular attention was paid to the choice of inhibitors and their concentrations to avoid non-specific effects common to enzyme inhibitors. We used two PKC inhibitors to assess the potential role of PKC isoforms in NECA-induced IL-6 release. Ro 31-8220 effectively inhibited NECA-induced formation of IL-6 at low concentrations (1 μM). At ≤1 μM, Ro 31-8220 is con-

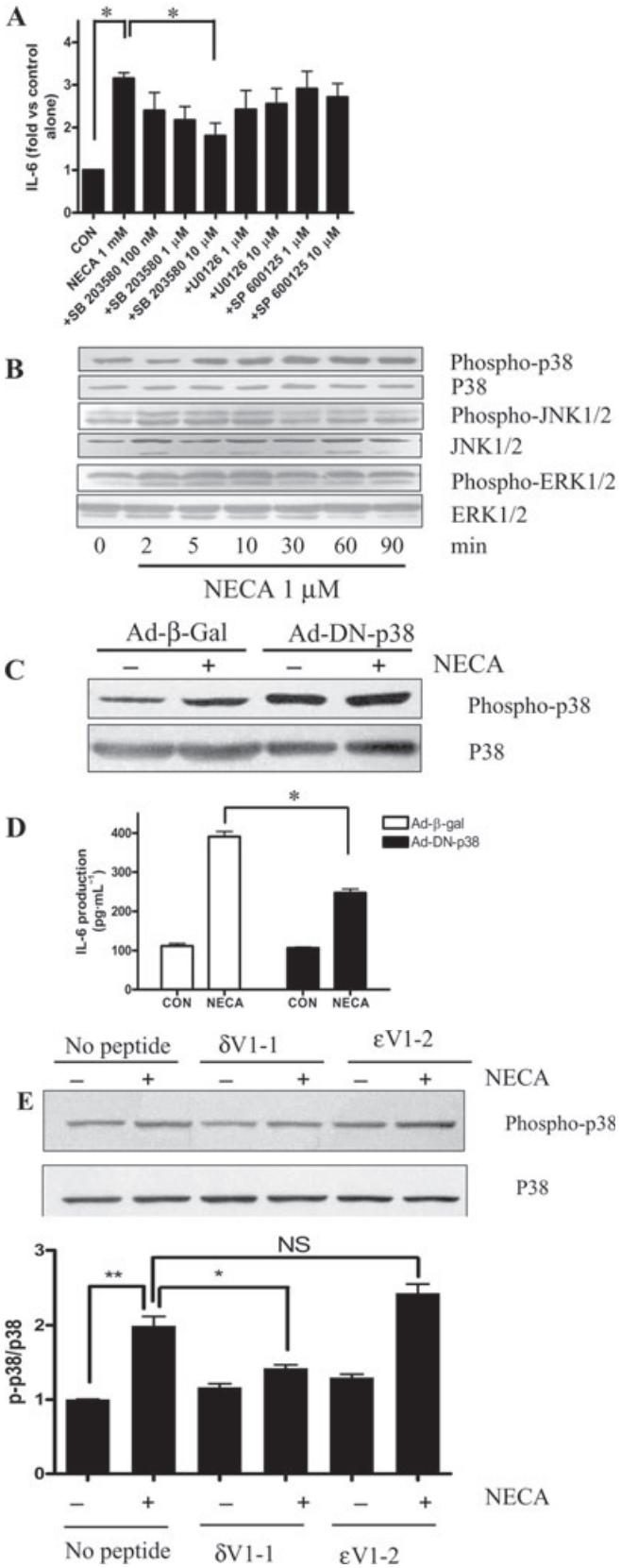

Figure 5 p38 MAPK is involved in NECA-induced IL-6 release. (A) CFs were pre-incubated with three MAPKs inhibitors at the indicated concentrations for 30 min, then stimulated with 1 μM NECA for 6 h. IL-6 in the supernatant was quantified by ELISA. Data are expressed as mean ± SD of three independent experiments. *$P < 0.05$ (B) After cell cultures had been deprived of serum for 24 h, the cells were treated with 1 μM NECA for 2–90 min. The level of phosphorylation of three MAPKs was determined by Western blot analysis. (C) Levels of phosphorylated and total p38 MAPK were measured after stimulation with 1 μM NECA for 15 min in CFs infected with recombinant adenovirus Ad-β-gal or Ad-DN-p38. (D) IL-6 content in the supernatant was measured after stimulation with1 μM NECA for 6 h in CFs infected with recombinant adenovirus Ad-β-gal or Ad-DN-p38. Data are expressed as mean ± SD of three independent experiments. *$P < 0.05$. (E) CFs were pre-incubated with the PKC-δ-selective inhibitor peptide (δV1-1, 5 mM) or the PKC-ε-selective inhibitor peptide (εV1-2, 5 mM) or no peptide for 60 min, and then stimulated with 1 μM NECA for 10 min, then levels of phosphorylated and total p38 MAPK were measured. Data are expressed as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$.

sidered to be a general PKC-specific, but not PKC isoform-selective, inhibitor, although it has been found to have less activity against atypical PKCs at this concentration (Yeo and Exton, 1995). However, GÖ 6976, with specificity for the PKC classical isoforms (α, βI, βII and γ) in the concentration range 1–100 nM (Martiny-Baron *et al*., 1993) had little influence on NECA-induced IL-6 release. Therefore, because of the uncertain specificity of these chemical inhibitors, we used the PKC subtype-selective translocation peptide developed by Mochly-Rosen to clarify the role of a specific PKC isoform in our experiments (Chen *et al*., 2001), and found that myristoylated δV1-1 inhibited the NECA-induced translocation of PKCδ (Supporting Information Figure S3). Furthermore, IL-6 release by NECA stimulation was greatly inhibited in the presence of the PKC-δ isoform translocation inhibitor peptide, but not by the PKC-ε translocation inhibitor peptide. Therefore, NECA-induced IL-6 release is dependent on PKC-δ. We subsequently confirmed that NECA induced phosphorylation on two sites of PKC-δ so as to activate PKC-δ. It should be noted that we did not preclude the possibility that other PKC isoforms (βI, βII, ζ) participate in the release of IL-6 induced by NECA in CFs.

We also investigated the roles of the MAPKs including ERK, JNK and p38 in the mechanism of NECA-stimulated IL-6 release. Inhibition of p38 MAPK by SB203580 dose-dependently suppressed NECA-induced IL-6 release. p38 MAPK has been found to mediate the effects of A_{2B} receptor stimulation in other cell models. Feoktistov *et al*. (1999) indicated that the p38 MAPK pathway is essential for adenosine A_{2B}R-dependent stimulation of IL-8 production in HMC-1 cells, and Fiebich *et al*. (2005) reported that p38 MAPK is crucial for the NECA-induced signalling cascade that leads to increased IL-6 synthesis in U373 MG cells. We also demonstrated that a PKC-δ isoform translocation inhibitor peptide, but not a PKC-ε inhibitor peptide, inhibited NECA-stimulated p38 MAPK phosphorylation, which indicates that PKC-δ functions as an upstream regulator of p38 MAPK in this process. However, this finding differs from others suggesting that NECA-stimulated p38 MAPK is cAMP dependent (Schulte and Fredholm, 2003); therefore, we presume that the mechanism through which activation of the A_{2B} receptor stimulates p38 MAPK differs substantially in different cell types.

In conclusion, we showed that the activation of adenosine receptors increases the expression and release of IL-6 in a time- and concentration-dependent manner in adult mouse CFs; in particular, this effect is mediated by A_{2B} receptors requiring the activation of the PKC-δ–p38 pathway by a mechanism that appears to be independent of cyclic AMP, PKA, JNK and ERK.

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References

Alexander SP, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edn. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Ashton KJ, Peart JN, Morrison RR, Matherne GP, Blackburn MR, Headrick JP (2007). Genetic modulation of adenosine receptor function and adenosine handling in murine hearts: insights and issues. *J Mol Cell Cardiol* **42** (4): 693–705.

Carapau D, Kruhofer M, Chatalbash A, Orengo JM, Mota MM, Rodriguez A (2007). Transcriptome profile of dendritic cells during malaria: cAMP regulation of IL-6. *Cell Microbiol* **9** (7): 1738–1752.

Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D *et al*. (2001). Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. *Proc Natl Acad Sci USA* **98** (20): 11114–11119.

Chio CC, Chang YH, Hsu YW, Chi KH, Lin WW (2004). PKA-dependent activation of PKC, p38 MAPK and IKK in macrophage: implication in the induction of inducible nitric oxide synthase and interleukin-6 by dibutyryl cAMP. *Cell Signal* **16** (5): 565–575.

Epperson SA, Brunton LL, Ramirez-Sanchez I, Villarreal F (2009). Adenosine receptors and second messenger signaling pathways in rat cardiac fibroblasts. *Am J Physiol Cell Physiol* **296** (5): C1171–C1177.

Evans BA, Elford C, Pexa A, Francis K, Hughes AC, Deussen A *et al*. (2006). Human osteoblast precursors produce extracellular adenosine, which modulates their secretion of IL-6 and osteoprotegerin. *J Bone Miner Res* **21** (2): 228–236.

Feoktistov I, Biaggioni I (1997). Adenosine A_{2B} receptors. *Pharmacol Rev* **49** (4): 381–402.

Feoktistov I, Goldstein AE, Biaggioni I (1999). Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. *Mol Pharmacol* **55** (4): 726–734.

Fiebich BL, Biber K, Gyufko K, Berger M, Bauer J, van Calker, D (1996). Adenosine A2b receptors mediate an increase in interleukin (IL)-6 mRNA and IL-6 protein synthesis in human astroglioma cells. *J Neurochem* **66** (4): 1426–1431.

Fiebich BL, Akundi RS, Biber K, Hamke M, Schmidt C, Butcher RD *et al*. (2005). IL-6 expression induced by adenosine A_{2B} receptor stimulation in U373 MG cells depends on p38 mitogen activated kinase and protein kinase C. *Neurochem Int* **46** (6): 501–512.

Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53** (4): 527–552.

Funaya H, Kitakaze M, Node K, Minamino T, Komamura K, Hori M (1997). Plasma adenosine levels increase in patients with chronic heart failure. *Circulation* **95** (6): 1363–1365.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B (1998). A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA* **95** (5): 2509–2514.

Jacobson KA, Gao ZG (2006). Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* **5** (3): 247–264.

Kanda T, Takahashi T (2004). Interleukin-6 and cardiovascular diseases. *Jpn Heart J* **45** (2): 183–193.

Kim YC, Ji X, Melman N, Linden J, Jacobson KA (2000). Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A(2B) adenosine receptors. *J Med Chem* **43** (6): 1165–1172.

Kiriyama Y, Murayama T, Tokumitsu Y, Nomura Y (1997). Protein kinase A-dependent IL-6 production induced by calcitonin in human glioblastoma A172 cells. *J Neuroimmunol* **76** (1–2): 139–144.

MacGowan GA, Mann DL, Kormos RL, Feldman AM, Murali S (1997). Circulating interleukin-6 in severe heart failure. *Am J Cardiol* **79** (8): 1128–1131.

Martiny-Baron G, Kazahietz MG, Mischak H, Blumberg PM, Kochs G, Hug H *et al*. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem* **268** (13): 9194–9197.

Nelson AR, Borland L, Allbritton NL, Sims CE (2007). Myristoyl-based transport of peptides into living cells. *Biochemistry* **46** (51): 14771–14781.

Ohnishi A, Li P, Branch RA, Biaggioni IO, Jackson EK (1988). Adenosine in renin-dependent renovascular hypertension. *Hypertension* **12** (2): 152–161.

Rees DA, Lewis BM, Lewis MD, Francis K, Scanlon MF, Ham J (2003). Adenosine-induced IL-6 expression in pituitary folliculostellate cells is mediated via A2b adenosine receptors coupled to PKC and p38 MAPK. *Br J Pharmacol* **140** (4): 764–772.

Roig E, Orus J, Pare C, Azqueta M, Filella X, Perez-Villa F *et al*. (1998). Serum interleukin-6 in congestive heart failure secondary to idiopathic dilated cardiomyopathy. *Am J Cardiol* **82** (5): 688–690, A8.

Ryzhov S, Zaynagetdinov R, Goldstein AE, Novitskiy SV, Blackburn MR, Biaggioni I *et al*. (2008). Effect of A2B adenosine receptor gene ablation on adenosine-dependent regulation of proinflammatory cytokines. *J Pharmacol Exp Ther* **324** (2): 694–700.

Schulte G, Fredholm BB (2003). The G(s)-coupled adenosine A(2B) receptor recruits divergent pathways to regulate ERK1/2 and p38. *Exp Cell Res* **290** (1): 168–176.

Schwaninger M, Neher M, Viegas E, Schneider A, Spranger M (1997). Stimulation of interleukin-6 secretion and gene transcription in primary astrocytes by adenosine. *J Neurochem* **69** (3): 1145–1150.

Tsutamoto T, Hisanaga T, Wada A, Maeda K, Ohnishi M, Fukai D *et al*. (1998). Interleukin-6 spillover in the peripheral circulation increases with the severity of heart failure, and the high plasma level of interleukin-6 is an important prognostic predictor in patients with congestive heart failure. *J Am Coll Cardiol* **31** (2): 391–398.

Vazquez JF, Clement HW, Sommer O, Schulz E, van Calker D (2008). Local stimulation of the adenosine A2B receptors induces an increased release of IL-6 in mouse striatum: an *in vivo* microdialysis study. *J Neurochem* **105** (3): 904–909.

Wan S, DeSmet JM, Barvais L, Goldstein M, Vincent JL, LeClerc JL (1996). Myocardium is a major source of proinflammatory cytokines in patients undergoing cardiopulmonary bypass. *J Thorac Cardiovasc Surg* **112** (3): 806–811.

Ward NE, O'Brian CA (1993). Inhibition of protein kinase C by N-myristoylated peptide substrate analogs. *Biochemistry* **32** (44): 11903–11909.

Yamada K, Goto A, Ishii M, Yoshioka M, Matsuoka H, Sugimoto T (1992). Plasma adenosine concentrations are elevated in conscious spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol* **19** (8): 563–567.

Yamada K, Goto A, Ishii M, Yoshioka M, Sugimoto T (1995). Plasma adenosine concentrations are elevated in Dahl salt-sensitive rats. *Experientia* **51** (3): 227–229.
Yeo EJ, Exton JH (1995). Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells. *J Biol Chem* **270** (8): 3980–3988.
Yin F, Li P, Zheng M, Chen L, Xu Q, Chen K *et al*. (2003). Interleukin-6 family of cytokines mediates isoproterenol-induced delayed STAT3 activation in mouse heart. *J Biol Chem* **278** (23): 21070–21075.
Zhao LJ, Jian H, Zhu H (2003). Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. *Gene* **316**: 137–141.
Zhong H, Belardinelli L, Maa T, Feoktistov I, Biaggioni I, Zeng D (2004). A(2B) adenosine receptors increase cytokine release by bronchial smooth muscle cells. *Am J Respir Cell Mol Biol* **30** (1): 118–125.
Zhong H, Belardinelli L, Maa T, Zeng D (2005). Synergy between A2B adenosine receptors and hypoxia in activating human lung fibroblasts. *Am J Respir Cell Mol Biol* **32** (1): 2–8.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Mouse CFs express four adenosine receptor subtypes. RT–PCR using primers for mouse A_1, A_{2A}, A_{2B} or A_3 adenosine receptors was performed without (lane 1; negative control) or with mRNA isolated from CFs (lanes 2–5). The PCR products of mouse A_1, A_{2A}, A_{2B} or A_3 ARs were accordingly 267, 122, 240 and 189 base pairs. Products were visualized by ethidium bromide staining, and compared to a 25-base pair ladder (lane M).

Figure S2 CFs were stimulated with NECA or forskolin at the indicated concentrations for 10 min. cAMP accumulation was determined as described under Methods. Data are given as % conversion of incorporated tritium into [^{3}H]-cAMP. $n = 3$, $^{**}P < 0.01$.

Figure S3 Western blot analysis of cytosolic and particulate fractions from adult mouse CFs was carried out as described to demonstrate isozyme-selective effects on PKCδ translocation (P/S). Cells were treated with NECA in the presence of δV1-1 (5 mM) or εV1-2 (5 mM). Soluble (S) and particulate (P) fractions were probed with anti-δPKC. $n = 3$, $^{**}P < 0.01$.

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