Extracellular adenosine 5′-diphosphate promotes MCP-1/CCL2 expression via the P2Y13 purinergic receptor/ ERK signaling axis in temporomandibular joint-derived mouse fibroblast-like synoviocytes

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Abstract

Background Temporomandibular joint osteoarthritis (TMJ-OA) causes cartilage degeneration, bone cavitation, and fibrosis of the TMJ. However, the mechanisms underlying the fibroblast-like synoviocyte (FLS)-mediated inflammatory activity in TMJ-OA remain unclear.

Methods and Results Reverse transcription-quantitative polymerase chain reaction analysis revealed that the P2Y1, P2Y12, and P2Y13 purinergic receptor agonist adenosine 5'-diphosphate (ADP) significantly induces monocyte chemotactic protein 1 (MCP-1)/C-C motif chemokine ligand 2 (CCL2) expression in the FLS1 synovial cell line. In contrast, the uracil nucleotide UTP, which is a P2Y2 and P2Y4 agonist, has no significant effect on MCP-1/CCL2 production in FLS1 cells. In addition, the P2Y13 antagonist MRS2211 considerably decreases the expression of ADP-induced MCP-1/CCL2, whereas ADP stimulation enhances extracellular signal-regulated kinase (ERK) phosphorylation. Moreover, it was found that the mitogen-activated protein kinase/ERK kinase (MEK) inhibitor U0126 reduces ADP-induced MCP-1/CCL2 expression.

Conclusions ADP enhances MCP-1/CCL2 expression in TMJ FLSs via P2Y13 receptors in an MEK/ERK-dependent manner, thus resulting in inflammatory cell infiltration in the TMJ. Collectively, the findings of this study contribute to a partial clarification of the signaling pathway underlying the development of inflammation in TMJ-OA and can help identify potential therapeutic targets for suppressing ADP-mediated purinergic signaling in this disease.

Introduction

The temporomandibular joint (TMJ) comprises the mandibular condyle and fossa [1], the osteoarthritis (OA) of which is characterized by bone cavitation, cartilage deterioration, and fibrosis, ultimately resulting in TMJ pain and stiffness [2]. Previous histological studies revealed the pathogenesis of fibrosis in TMJ-OA [3, 4], indicating that the formation of fibrotic tissue can cause restricted joint movements [5]. Moreover, Zang et al. demonstrated that chronic aseptic inflammation is associated with macrophage pyroptosis that contributes to fibrosis [6].

Compared to autophagy and apoptosis, necroptosis leads to pronounced detrimental effects on tissue homeostasis by promoting the release of damage-associated molecular patterns (DAMPs), including nucleotides, high mobility group box 1, and uric acid [7]. However, endogenous ligands of purinergic receptors, such as extracellular adenine nucleotide (ATP) and adenosine 5'-diphosphate (ADP), which function as paracrine signaling molecules, have considerable potential in both pathological and physiological control of such processes [8]. Zhou et al. reported that platelet-derived ADP functions as an important mediator that promotes chondrocyte-based cartilage repair and proliferation in OA [9]. In addition, ATP and uracil nucleotide (UTP) have been shown to stimulate calcium mobilization from intercellular stores in human rheumatoid synovial cells [10].
We previously established that the fibroblast-like synoviocyte synovial cell line FLS1 (obtained from the TMJ of mouse) exhibits myofibroblastic characteristics [11]. We also demonstrated that FLS1 cells express several subtypes of the P2 receptor [12]. Typically, P2X receptors comprise ion channels that mediate the passage of cations, such as sodium, potassium, or calcium; P2Y receptors couple with the G proteins involved in the modulation of cytoplasmic Ca\(^{2+}\) concentrations and regulation of intracellular adenylyl cyclase [13]. Moreover, Jacobson et al. reported that ATP activates P2X receptors and that five or more nucleotides (including ATP, ADP, and UTP) activate P2Y receptors [14], whereas in macrophage lineage cells, extracellular ADP has also been found to promote the expression of monocyte chemotactic protein 1 (MCP-1)/C-C motif chemokine ligand 2 (CCL2) via extracellular signal-regulated kinase (ERK) signaling [15].

Chemokines play major roles in the selective recruitment of neutrophils, lymphocytes, and monocytes as well as in the activation of G protein-coupled receptors, among which MCP-1/CCL2 regulates the infiltration and migration of monocytes/macrophages [16]. However, it is yet to be established whether extracellular nucleotides affect the expression levels of MCP-1/CCL2 in FLSs.

In this study, we examined the mechanism by which DAMPs, such as ATP, ADP, and UTP, influence the expression of MCP-1/CCL2 in FLS1 cells. Specifically, we assessed whether ATP, ADP, and UTP affect the activity of mitogen-activated protein kinases (MAPKs), such as ERK, p38 MAPK, and c-Jun N-terminal kinase (JNK), in FLS1 cells. Moreover, we investigated whether ATP-, ADP-, or UTP-activated MAPKs influence the status of MCP-1/CCL2 expression in FLS1 cells. Our findings contribute to a better understanding of the signaling pathway that underlies the development of inflammation in TMJ-OA, and provide important insights regarding the potential therapeutic significance of TMJ-OA-related inflammatory activity.

**Materials And Methods**

**Reagents**

ATP, ADP, and UTP were acquired from Merck KGaA (Darmstadt, Germany). Antagonists of P2Y\(_1\) (MRS 2179), P2Y\(_{12}\) (AR-C 66096), and P2Y\(_{13}\) (MRS 2211) were purchased from R&D Systems (Minneapolis, MN, USA), and U0126 and the p38 MAPK inhibitor SB203580 were purchased from Calbiochem (Merck KGaA).

**Cell culture**

FLS1 cells were cultured in Ham's F-12 medium containing 10% FBS (Invitrogen, Carlsbad, CA, USA), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. To evaluate MCP-1/CCL2 mRNA expression, these cells were treated with different concentrations of ATP, ADP, and UTP for the indicated time periods. Prior to ADP administration, the cells were pre-treated for 30 min with P2Y purinergic receptor antagonists MRS 2179, AR-C 66096, and MRS 2211 to evaluate the effects of these antagonists.
on the ADP-induced promotion of MCP-1/CCL2 expression. In addition, prior to ADP stimulation, some cells were pre-treated for 30 min with U0126 or SB203580.

**RNA isolation and reverse transcription-quantitative polymerase chain reaction**

FLS1 cells were seeded in 12-well plates (7 × 10⁴ cells/well) containing Ham's F-12 medium for 24 h, and were thereafter cultured with or without ATP, ADP, or UTP for 24 h. Following incubation, total RNA was isolated from cells using ISOGEN reagent (Nippon Gene, Toyama, Japan). Complementary DNA was synthesized from 1 µg of the isolated RNA using a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan) and used as a template for PCR performed using SYBR Premix Ex Taq II (Takara Bio) in a Thermal Cycler Dice Real Time System (Takara Bio). Amplifications were performed using the sequence-specific mouse *MCP-1* oligonucleotide primers 5′-AGCAGCAGGTGCTCCAAGA-3′ (forward) and 5′-GTGCTGAAGACCTTAGGGCAGA-3′ (reverse). As an internal control gene, we used mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which was amplified using the primer pair 5′-TGTGCCTGCTGGATCTG-3′ (forward) and 5′-TTGCTGGTTGAGTCGAGGAG-3′ (reverse).

**Western blot analysis**

FLS1 cells were seeded in 10-cm tissue culture plates at 1 × 10⁶ cells/10 cm, and thereafter cultured in serum-free Ham's F-12 medium for 24 h. The cells were cultured with or without ADP, with some groups being pre-treated with U0126 for the indicated times. Following incubation, total protein was extracted from the cells using RIPA lysis buffer (Sigma-Aldrich) containing protease and phosphatase inhibitor cocktails (Sigma). The protein content of each sample was measured using BCA reagent (Pierce, Rockford, IL, USA). Extracts containing equal amounts of protein were loaded on 12.5% SDS-PAGE gels, and the separated proteins were subsequently transferred to PVDF membranes and blocked with 5% skim milk in Tris-buffered saline containing Tween 20 (TBS-T) (Takara, Bio) for 1 h. The blocked membranes were then incubated overnight at 4 °C with the following primary antibodies obtained from Cell Signaling Technology, Beverly, MA, USA: ERK1/2 (#9102), phospho-ERK1/2 (T202/Y204, #9101), p38 MAPK (#9212), phospho-p38 MAPK (T180/Y182, #9211), stress-activated protein kinase (SAPK)/JNK (#9252), phospho-SAPK/JNK (T183/Y185, #9251), and GAPDH (D16H11, #5174). The following day, the membranes were incubated with appropriate secondary antibodies for 1 h, and bound antibodies were visualized using an alkaline phosphatase substrate kit (BCIP/NBT Substrate Kit; Vector Laboratories Inc., Burlingame, CA, USA). Expression of the phosphorylated target proteins was quantified using ImageJ 1.53a software (Wayne Rasband, NIH, USA).

**Statistical analysis**

All experiments were performed in triplicate. The data are presented as the means ± standard deviation (n = 3). Statistical significance was evaluated using Tukey’s multiple comparison test using SPSS software (version 24.0). P-values were considered significant if *P* < 0.01 and **P** < 0.05.
Results

ADP is more effective than ATP in promoting the expression of MCP-1/CCL2 mRNA in FLSs

As shown in Fig. 1, compared to the control cells, cells treated with ATP and ADP (100 µM) showed 1.57-fold and 2.31-fold upregulation of MCP-1/CCL-2 mRNA expression in FLS1 cells, respectively, thereby indicating greater efficacy of ADP over ATP. In contrast, treatment with UTP (100 µM) was found to have no discernable effect on the expression of MCP-1/CCL2 mRNA in FLS1 cells.

P2Y purinergic receptor antagonists suppress the ADP-promoted mRNA expression of MCP-1/CCL2 in FLSs

As shown in Figs. 2A and 2B, treatment with neither MRS2179 (100 µM) nor ARC-66096 (100 µM) had any appreciable effect on the ADP-promoted mRNA expression of MCP-1/CCL2 in FLS1 cells. However, MRS 2211 (50–100 µM) was observed to significantly reduce the ADP-promoted expression of MCP-1/CCL2 mRNA in a concentration-dependent manner (Fig. 2C).

ADP upregulates ERK1/ERK2 phosphorylation in FLSs

Using western blotting, we evaluated the phosphorylation status of ERK1/2 following stimulation of FLS1 cells with ADP. As shown in Fig. 3A, ADP (100 µM) treatment led to an upregulation of ERK1/2 phosphorylation, with peak expression levels being detected 10 min after the treatment, following which, expression levels declined between 30 to 120 min post-treatment. Moreover, we established that such ADP-promoted upregulation of ERK1/2 phosphorylation could be abrogated by the administration of U0126 (10 µM) (Fig. 3B). We also confirmed that ADP (100 µM) had no significant effects on p38 MAPK phosphorylation levels and that SAPK/JNK remained undetectable in FLS1 cells, even after ADP stimulation (data not shown).

The MEK inhibitor U0126 suppresses ADP-promoted mRNA expression of MCP-1/CCL2 in FLSs

We also verified that U0126 (10 µM) (Fig. 4A) and SB 203580 (10 µM) (Fig. 4B) completely abrogated the ADP-promoted expression of MCP-1/CCL2 mRNA.

Discussion

In this study, we demonstrated that extracellularly applied ADP is more effective than ATP in promoting the expression of MCP-1/CCL2 in FLSs derived from the mouse TMJ (Fig. 1), thereby providing evidence to indicate that P2X receptor-mediated signaling might also positively regulate MCP-1/CCL2 expression in FLSs. Given that the intercellular mechanisms underlying the ATP-mediated induction of MCP-1/CCL2
expression have yet to be identified, elucidating the mechanisms associated with ATP stimulation in FLS1 cells will be the focus of our future studies. However, we established herein that UTP has no appreciable effects on MCP-1/CCL2 expression in FLS1 cells (Fig. 1). In our previous studies, we demonstrated that UTP significantly reduces mRNA expression of the fibrogenic marker α-SMA in FLS1 cells [12]. In general, UTP preferentially binds to and activates P2Y_2, P2Y_4, and P2Y_6 receptors [13], and FLS1 cells strongly express P2Y_2, P2Y_4, P2Y_12, P2Y_13, and P2Y_14 receptors [12], thereby indicating that UTP enhances the expression of α-SMA mRNA via interaction with either P2Y_2 or P2Y_4 receptors in these cells. Altogether, these findings indicate that UTP-induced specific intracellular signaling mediated by P2Y_2 and P2Y_4 receptors does not enhance the expression of MCP-1/CCL2 in FLS1 cells.

Given that ADP typically binds to P2Y_1, P2Y_12, and P2Y_13, we sought to determine which subtypes of P2Y receptor mediate the promotive function of ADP in MCP-1/CCL2 expression. In this regard, we found that the P2Y_1 antagonist MRS2179 (10–100 µM) had no effect on the ADP-promoted expression of MCP-1/CCL2 in FLSs (Fig. 2A). Previously, Bynagari et al. demonstrated that MRS2179 (100 µM) significantly downregulates the 2MeSADP-induced phosphorylation of nPKCeta in human platelets [17], and Atterbury-Thomas et al. reported that MRS2179 (10 µM) suppresses the ADP-induced calcium increase in mouse glial cells [18]. These findings together indicate that MRS2179 concentrations in the range 10–100 µM would be optimal for antagonizing ADP-activated P2Y_1 signaling. In addition, we also showed that the P2Y_12 antagonist ARC-66096 (10–100 µM) had no effects on the ADP-promoted expression of MCP-1/CCL2 in FLSs (Fig. 2B). Bélanger et al. reported that ARC-66096 (10 µM) significantly inhibits platelet aggregation [19], and Quintas et al. demonstrated that ARC-66096 (10 µM) can significantly abrogate adenosine 5′-O-(2-thio)-diphosphate-induced astroglial proliferation [20], thus indicating 10 µM to be the optimal concentration of ARC-66096 for antagonizing ADP-activated P2Y_12 signaling. Notably, we established that the P2Y_13 antagonist MRS2211 (50–100 µM) significantly abrogated the ADP-promoted expression of MCP-1/CCL2 in a concentration-dependent manner (Fig. 2C). In contrast, Kim et al. demonstrated that when applied at a concentration of 30 µM, MRS2211 inhibited ADP-induced inositol triphosphate production in human astrocytoma cells [21]. In addition, Zeng et al. demonstrated that MRS2211 (100 µM) abrogated ADP-induced Ca^{2+} mobilization in primary cultured microglia [22]. Therefore, the application MRS2211 in the concentration range 30–100 µM is optimal for antagonizing ADP-activated P2Y_13 signaling. Collectively, these findings provide convincing evidence to indicate that extracellularly applied ADP promotes the expression of MCP-1/CCL2 mRNA via the activation of P2Y_13 receptors in FLS1 cells.

Earlier, Inose et al. demonstrated that levels of MCP-1/CCL2 mRNA expression and those of its receptor CCR2 were significantly increased in response to lysophosphatidylcholine (LPC) stimulation, and that the LPC-mediated increase in MCP-1/CCL2 transcripts was reduced by blocking the P2X receptor P2X_7 in a microglial-derived cell line [23]. Moreover, Satonaka et al. reported that ADP upregulated the expression of MCP-1/CCL2 mRNA in cultured rat vascular smooth muscle cells (VSMCs), which was significantly inhibited by a P2Y_12 inhibitor, thereby indicating that ADP promotes MCP-1/CCL2 mRNA expression via
P2Y12 receptors in VSMCs [24]. Taken together, these observations imply that intracellular mechanisms underlying the extracellular nucleotide-mediated regulation of MCP-1/CCL2 expression may differ according to cell type.

In the present study, we established that ADP also promotes ERK1/2 signaling in FLS1 cells (Fig. 3A) and confirmed that U0126 abrogates this ADP-mediated upregulation of ERK1/2 phosphorylation (Fig. 3B). Moreover, U0126 partially (nevertheless significantly) abrogated the ADP-mediated upregulation of MCP-1/CCL2 mRNA expression (Fig. 4A), thereby providing convincing evidence that ADP promotes MCP-1/CCL2 mRNA expression in FLSs in an MEK/ERK-dependent manner, which positively regulates the infiltration of monocytes/macrophages into the mouse TMJ.

Previously, Liao et al. demonstrated that the expression of interleukin-17 (IL-17), which plays an essential role in the immune system and in the development of infectious and inflammatory diseases, upregulates MCP-1/CCL2 in RAW264.7 cells via p38 MAPK [25]. Moreover, Satonaka et al. reported that JNK/SAPK inhibition attenuates the ADP-induced upregulation of MCP-1/CCL2 mRNA and protein in VSMCs [24], whereas Ip et al. demonstrated that IL-1 and IL-13 induce MCP-1/CCL2 expression in ERK- and p38 MAPK-dependent manners in human bronchial epithelial cells [26]. Moreover, Wuyts et al. reported that IL-1β promotes the expression of MCP-1/CCL2 protein in ERK, p38 MAPK, and JNK/SAPK-dependent manners in human airway smooth muscle cells [27]. Collectively, these findings suggest that MAPK (ERK, p38 MAPK, and JNK/SAPK)-mediated intracellular signaling plays important roles in the expression of MCP-1/CCL2. However, in the present study, western blot analysis enabled us to confirm that ADP (100 µM) does not significantly affect the phosphorylation of p38 MAPK in FLS1 cells, and we failed to detect JNK/SAPK, even in response ADP stimulation (data not shown). These findings, therefore, provide a strong indication that at least in case of FLSs, ADP does not promote the expression of MCP-1/CCL2 via p38 MAPK or JNK/SAPK signaling pathways. We also established that the p38 inhibitor SB 203580 abrogates the ADP-promoted mRNA expression of MCP-1/CCL2 (Fig. 4B), thereby indicating that the basal activity of p38 MAPK plays an important role in the ADP-induced promotion of MCP-1/CCL2 expression in FLS1 cells.

Based on the findings of this study, we identified inflammatory molecules underlying the development of inflammation in TMJ-OA, which in turn enabled us to establish the potential therapeutic significance of TMJ-OA-related inflammatory activity. Herein, we provide convincing evidence to indicate that ADP might serve as an effective molecular target for preventing OA-related inflammation around the TMJ.

**Declarations**

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**Author contributions** SJ performed and interpreted data from RT-qPCR and western blot analysis, and was a contributor in writing the manuscript. NC was a contributor in writing the manuscript. SM was a contributor in assisting SY in performing RT-qPCR and western blot analysis. KS was a contributor in
designing the experimental plan. AI made substantial contributions to the conception and design of the study, and was a contributor in writing the manuscript. All authors read and approved the final manuscript.

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**Conflict of Interest** The authors declare that they have no competing interests.

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent to publication** Not applicable.

**References**


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**Figures**

![Bar graph showing the relative expression of MCP-1/GAPDH](image-url)
**Figure 1**

*ADP promotes the expression of MCP-1/CCL2 in fibroblast-like synoviocytes.* Cells were cultured with or without ATP (100 μM), ADP (100 μM), or UTP (100 μM) for 24 h. The relative expression of MCP-1/CCL2 was determined using RT-qPCR. Data are presented as the means ± standard deviation (SD) (n = 3). *P < 0.01.

**Figure 2**

*MSR 2211 concentration-dependently suppresses the expression of MCP-1/CCL2 in fibroblast-like synoviocytes.* Cells were cultured with or without ADP (100 μM) for 24 h. Cells were pre-treated with the (A) MRS 2179, (B) AR-C 66096, or (C) MRS 2211 for 30 min prior to stimulation at the indicated concentrations. The relative expression of MCP-1/CCL2 was determined using RT-qPCR. Data are presented as the mean ± SD (n = 3). *P < 0.01. **P < 0.05.

**Figure 3**

*ADP upregulates ERK-1/2 phosphorylation in fibroblast-like synoviocytes.* Cells initially were starved and then treated (A) with ADP (100 μM) for the indicated times or (B) with or without ADP (100 μM) for 30 min. In the latter case, selected cell groups were pre-treated with U0126 (10 μM) for 30 min prior to stimulation with ADP (100 μM). ERK1/2 phosphorylation was evaluated based on western blot analysis. Data are presented as the means ± SD (n = 3). *P < 0.01. **P < 0.05.

**Figure 4**

*U0126, suppresses the ADP-promoted expression of MCP-1/CCL2 in fibroblast-like synoviocytes.* Cells were cultured with ADP (100 μM) for 24 h. Selected cell groups were pre-treated with (A) U0126 (10 μM) or (B) SB203580 (10 μM) for 30 min prior to stimulation. The relative expression of MCP-1/CCL2 were determined using RT-qPCR. Data are presented as the means ± SD (n = 3). *P < 0.01. **P < 0.05.