Modafinil exerts anti-inflammatory and anti-fibrotic effects by upregulating adenosine A\textsubscript{2A} and A\textsubscript{2B} receptors

Haiyan Li  
Ewha Womans University Medical Center

Ji Aee Kim  
Ewha Womans University Medical Center

Seong-Eun Jo  
Ewha Womans University Medical Center

Huisu Lee  
Ewha Womans University Medical Center

Kwan-Chang Kim  
Ewha Womans University Medical Center

Shinkyu Choi  
Ewha Womans University Medical Center

Suk Hyo Suh (shsuh@ewha.ac.kr)  
Ewha Womans University Medical Center

Research Article

Keywords: fibrosis, lung and liver, A2A and A2B adenosine receptors, Epac signalling

Posted Date: February 23rd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2606041/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Purinergic Signalling on November 8th, 2023. See the published version at https://doi.org/10.1007/s11302-023-09973-8.
Abstract

Adenosine receptor (AR) suppresses inflammation and fibrosis by activating cyclic adenosine monophosphate (cAMP) signaling. We investigated whether altered AR expression contributes to the development of fibrotic diseases and whether $A_{2A}$ AR and $A_{2B}$ AR upregulation inhibits fibrotic responses. Murine models of fibrotic liver or pulmonary disease were developed by injecting thioacetamide intraperitoneally, by feeding a high-fat diet, or by intratracheal instillation of bleomycin. Modafinil was orally administered to inhibit fibrotic responses. The protein levels of $A_{2A}$ AR, $A_{2B}$ AR, and exchange protein directly activated by cAMP (Epac) were reduced, while collagen and α-smooth muscle actin (α-SMA) were elevated in diseased (idiopathic pulmonary fibrosis) human lung fibroblasts (HLFs) compared to normal HLFs. In liver or lung tissue from murine models of fibrotic diseases, $A_{2A}$ and $A_{2B}$ AR were downregulated, but $A_1$ and $A_3$ AR were not. Epac levels decreased, and levels of collagen, α-SMA, $K_{Ca}2.3$, and $K_{Ca}3.1$ increased compared to the control. Modafinil restored the levels of $A_{2A}$ AR, $A_{2B}$ AR, and Epac, and reduced collagen, α-SMA, $K_{Ca}2.3$, and $K_{Ca}3.1$ in murine models of fibrotic diseases. Transforming growth factor-β reduced the levels of $A_{2A}$ AR, $A_{2B}$ AR, and Epac, and elevated collagen, α-SMA, $K_{Ca}2.3$, and $K_{Ca}3.1$ in normal HLFs; however, these alterations were inhibited by modafinil. Our investigation revealed that downregulation of $A_{2A}$ AR and $A_{2B}$ AR reduced cAMP signaling and induced liver and lung fibrotic diseases while upregulation attenuated fibrotic responses, suggesting that $A_{2A}$ AR and $A_{2B}$ AR-upregulating agents, such as modafinil, may serve as novel therapies for fibrotic diseases.

Introduction

Fibrosis is a normal healing response to injury and is associated with the accumulation of extracellular matrix (ECM) proteins and other materials in the tissue. Additionally, it is a pathological feature of most chronic inflammatory diseases afflicting various organs such as the liver and lungs [1]. When injury is repetitive or severe, fibrotic response is upregulated and continuously producing ECM material such as collagen (Col) which thereby accumulates in tissue, which can lead to disruption of tissue architecture—deteriorating the functions of organ systems. Fibrotic diseases are estimated to cause over 800,000 deaths each year worldwide [2], and the incidence of fibrotic diseases will increase further with an increase in the aged population [2–4].

Idiopathic pulmonary fibrosis (IPF), the most common type of pulmonary fibrosis, is a condition in which the lungs become injured diffusely and progressively in the parenchyma, and has a poor prognosis with a median survival of only two to three years [5]. The adjusted incidence and prevalence of IPF was estimated to be in the range of 0.09–1.30 and 0.33–4.51 per 10,000 persons, respectively [6]. Chronic inflammatory liver diseases are caused by infection with hepatitis B or C virus, fat accumulation, or chronic exposure to hepatotoxic agents such as alcohol. In the United States, non-alcoholic fatty liver disease occurs in 25–30% of the general population and can progress to nonalcoholic steatohepatitis [7]. Liver fibrosis occurs in most chronic inflammatory diseases. Despite the high prevalence and clinical
importance of fibrotic diseases, their pathophysiology is yet to be elucidated, and anti-fibrotic therapeutics have not yet been developed.

Cyclic adenosine monophosphate (cAMP) signaling modulates inflammatory and fibrotic responses via an exchange protein activated by a cAMP (Epac) and protein kinase A (PKA)-mediated pathway [8–11]. The intermediate conductance Ca\(^{2+}\)-activated K\(^{+}\) channel K\(_{\text{Ca}}\)3.1 may play an important role in the modulation of inflammatory and fibrotic processes by cAMP. K\(_{\text{Ca}}\)3.1 regulates a variety of cellular processes involved in inflammatory and fibrotic processes, including cell activation, migration, and proliferation, by modulating Ca\(^{2+}\) signaling [12, 13]. K\(_{\text{Ca}}\)3.1 upregulation occurs in inflammatory and fibrotic responses and contributes to the development of fibrotic diseases. Conversely, K\(_{\text{Ca}}\)3.1 downregulation (or inhibition) attenuates inflammatory and fibrotic responses. Cyclic AMP downregulates K\(_{\text{Ca}}\)3.1 via an Epac-mediated pathway [12] and inhibits the K\(_{\text{Ca}}\)3.1 current through PKA-dependent phosphorylation of K\(_{\text{Ca}}\)3.1 [14]. Thus, cAMP-elevating agents such as modafinil (MF) [12, 14, 15] might modulate inflammation and fibrosis via Epac- and PKA-mediated pathways.

The adenosine receptors (ARs; A\(_{1}\), A\(_{2A}\), A\(_{2B}\), and A\(_{3}\)) are G-protein coupled receptors (GPCRs) that affect cellular cAMP levels [16]. A\(_{2A}\) AR and A\(_{2B}\) AR elevate cAMP levels via G\(_{s}\)-protein, whereas A\(_{1}\) AR and A\(_{3}\) AR decrease cAMP levels via G\(_{i}\) protein. A\(_{2A}\) AR-null mice are more susceptible to bleomycin (BLM)-induced lung injury [17], suggesting that A\(_{2A}\) AR play an important role in inhibiting pulmonary fibrosis in mice. A\(_{2B}\) AR is expressed in most inflammatory cells and has both proinflammatory and anti-inflammatory effects [18]. It generates anti-inflammatory effects when coupled with protein G\(_{s}\) and proinflammatory effects when coupled with G\(_{q}\) protein [19]. The predominant AR in cardiac fibroblasts is the A\(_{2B}\) AR, which modulates multiple processes in cardiac fibrosis [20]. Stimulation of A\(_{2B}\) AR inhibited myofibroblast differentiation and Col synthesis by pro-fibrotic agonists, such as transforming growth factor β (TGFβ) in cardiac fibroblasts. Thus, we examined whether reducing cAMP signaling by altering the expression of ARs contributes to the development of fibrotic diseases and whether the restoration of downregulated A\(_{2A}\) AR and A\(_{2B}\) AR could serve as a novel therapeutic approach for fibrotic diseases.

Materials And Methods

Chemicals

(S)-isomer of MF (MF-S) was synthesized by Cellion Biomed Inc. (Daejeon, South Korea), according to a published procedure [21]. Thioacetamide (TAA), bleomycin (BLM) and TGFβ were purchased from Sigma-Aldrich (St. Louis, MO). 1-(6-Amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuranuronamide (NECA) was from Tocris Bioscience (Minneapolis, MN). The other reagents used in the study were purchased from Sigma-Aldrich unless otherwise specified. MF was first dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was 0.5% (diluted in distilled water).

Murine model of NASH and MF-S treatment
A choline-deficient, L-amino-acid-defined, high-fat diet with 0.1% methionine (CDAHFD; A06071302, Research Diets, New Brunswick, NJ, USA) was used to induce steatosis, steatohepatitis, and hepatic fibrosis in mice [22]. Six-week-old C57BL/6 mice (18–22 g; n = 50; Oriental Bio, Seoul, Korea) were randomly divided into control and CDAHFD group and fed a standard laboratory murine diet and CDAHFD, respectively. The mice in these CDAHFD groups were fed a CDAHFD for 20 weeks.

Mice fed CDAHFD were randomly assigned to either vehicle- (CDAHFD group; n = 10) or MF-S-treated groups (CDAHFD + MF-S group; n = 30). MF-S was administered orally once daily, 5 times/week, and at four weeks after the start of CDAHFD for 16 weeks at doses of 10, 50, or 100 mg/kg (5–10/each dosage of MF-S). The control and CDAHFD groups were administered only the vehicle at the start of MF-S administration.

After 20 weeks, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight), and their livers and blood samples were collected for analysis.

**Murine model of toxic hepatitis and MF-S treatment**

TAA have been used to induce hepatitis and fibrosis. Six-week-old C57BL/6 male mice (n = 26) were randomly subdivided into two groups: control mice (n = 8) that received the vehicle for 16 weeks, and mice in the TAA-induced liver disease group (n = 35) that received TAA for 16 weeks.

Mice in the TAA-induced liver disease group were randomly assigned to either the vehicle- (TAA group; n = 13) or MF-S-treated groups (TAA + MF-S group; n = 25). MF-S was administered orally once daily and 5 times/week from 8 weeks after the start of TAA for 8 weeks at dosages of 10, 50, or 100 mg/kg (7–10/each dosage of MF-S). The control and CDAHFD groups were administered the vehicle at the start of MF-S administration.

TAA (100 mg/kg) was injected intraperitoneally three times per week, whereas MF-S was administered orally five times per week. Three mice in the TAA group were sacrificed eight weeks after TAA administration to confirm TAA-induced liver inflammation and fibrosis, while the remaining mice were sacrificed 16 weeks after TAA administration. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight), and their livers and blood samples were collected for analysis.

**Murine model of pulmonary fibrosis and MF-S treatment**

BLM has been used to induce pulmonary inflammation and fibrosis. Six-week-old C57BL/6 male mice (n = 50) were randomly subdivided into three groups: control mice (n = 10) that received the vehicle; mice in the BLM-induced pulmonary disease group (BLM group, n = 10) that received BLM (1.5 units/kg); and mice in the treatment groups (BLM + MF-S) that received BLM + MF-S for three weeks.

BLM (1.5 units/kg) was administered intratracheally once. MF-S (10, 50, or 100 mg/kg) was orally administered five times per week (n = 10/each per dosage) starting from one week after BLM instillation. The control and BLM groups were administered only the vehicle at the start of MF administration.
Four weeks after BLM instillation, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight); the lungs and blood samples were collected for analysis.

**Histopathology and immunohistochemistry**

The liver and lung specimens were fixed overnight in 10% formalin solution, then dehydrated, embedded in paraffin, and cut into 4-µm sections. Sections were stained with hematoxylin and eosin (H&E), Masson's trichrome (DAKO, Santa Clara, CA, USA), and Picro Sirius red stain kit (Abcam, Cambridge, UK). For immunohistochemical analysis, sections were incubated with primary antibodies against α-smooth muscle actin (α-SMA), and reticulin (Abcam) for 1 h at 37°C, then processed by an indirect immunoperoxidase technique using a commercial kit (DAKO). The slides were examined with a BX50 microscope (Olympus, Tokyo, Japan) and analyzed with the iSolution DT software (IMT i-Solution, Vancouver, BC, Canada).

**Cell culture and treatment of cells with reagents**

Primary human lung fibroblasts from a normal adult (NHLFs) and primary human lung fibroblasts from a patient with IPF (DHLFs) were purchased from Lonza (Basel, Switzerland) and cultured at 37°C in Fibroblast growth medium 2 (CC-3131; Lonza).

All cells were maintained at 37°C in a humidified condition under 5% CO₂. Media were changed twice weekly and cultures were passaged at a dilution of 1:2 weekly. The medium was then removed and replaced with fresh medium; the cells were maintained for the time periods indicated.

**Quantitative polymerase chain reaction (PCR)**

RNA isolation was performed using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was then reverse-transcribed using a high-capacity complementary DNA archive kit (Applied Biosystems, Foster City, CA, USA). PCR was performed on an ABI 7000 sequence detection system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Primer sets (Table 1) used for real-time PCR were designed using Primer3 (http://frodo.wi.mit.edu/primer3).

<table>
<thead>
<tr>
<th>primer</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCY2</td>
<td>5'-GAG CGG AGA GTA CTG GAT CG-3'</td>
<td>5'-GCT TCT TTT CCT TGG GGT TC-3'</td>
</tr>
<tr>
<td>ADCY3</td>
<td>5'- GGC TTC AGA GAC AAG GAA CG -3'</td>
<td>5'- TGG AGG GAT TGT TTC CTC TG -3'</td>
</tr>
<tr>
<td>ADCY9</td>
<td>5'- TCT TGA GCA AGC CGG ACT AT -3'</td>
<td>5'- CGA ACT CGA AGA GGA TAC GC -3'</td>
</tr>
<tr>
<td>ADCY10</td>
<td>5'- AAA AAC CTC GAC CAC CAC AG -3'</td>
<td>5'- TTT CTG CCC CTC AGA TAT GG -3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CTC CCA CTC TTC CAC CTT CG-3'</td>
<td>5'-TAG GGC CTC TCT TGC TCA GT-3'</td>
</tr>
</tbody>
</table>

**Western Blotting Analysis**
Tissue or cell extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 7.5–12% gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with Tris-buffered saline with 0.1% Tween-20 containing 5% bovine serum albumin at room temperature. The membranes were immunoblotted using antibodies against $A_1$ AR (Abcam), $A_{2A}$ AR (Abcam), $A_{2B}$ AR (Abcam) and $A_3$ AR (Abcam), $K_{Ca3.1}$ (Santa Cruz Biotechnology, Dallas, TX, USA), $K_{Ca2.3}$ (Santa Cruz Biotechnology), Col1 (Cell Signaling Technology Inc., Danvers, MA, USA), $\alpha$-SMA (Abcam), Epac1 (Santa Cruz Biotechnology), and Epac2 (Santa Cruz Biotechnology), and bands were visualized by chemiluminescence. Data processing was performed using a LAS-3000 luminescent image analyzer and IMAGE GAUSE software (Fuji Film, Tokyo, Japan).

**Procollagen type1 N-terminal propeptide (P1NP) measurement**

The P1NP levels were measured from mice sera using a P1NP enzyme-linked immunosorbent assay kit (#MBS2500076; MyBioSource, San Diego, CA, USA). The experimental procedure was performed according to the manufacturer's instructions.

**Hydroxyproline colorimetric assay**

Measurement of hydroxyproline in liver tissues was performed by manufacturer's instruction (MAK008, Sigma Aldrich). Briefly, tissues were homogenized and hydrolyzed for 3 h by adding concentrated hydroxychloride. Each hydrolyzed sample was transferred in a 96-well plate and dried under vacuum. After that, hydroxyproline concentration was determined by the reaction of oxidized hydroxyproline with 4-(Dimethylamino)benzaldehyde, which results is proportional to the hydroxyproline content.

**Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM) values. To examine the statistical significance among groups, one-way analysis of variance (ANOVA) with Bonferroni’s post hoc or two-tailed Student $t$-test was employed. Values with $P< 0.05$ were considered to be statistically significant.

**Results**

**The expression of ARs is altered in hepatic and pulmonary fibrotic diseases**

We compared the expression levels of $A_{2A}$ AR and $A_{2B}$ AR between normal human lung fibroblasts NHLFs and diseased human lung fibroblasts from IPF DHLFs. Protein levels of $A_{2A}$ AR and $A_{2B}$ AR were significantly reduced in DHLFs compared to those in NHLFs (Fig. 1a, left and middle panels). TGFβ, which plays a critical role in inflammation and fibrosis, affects the expression levels of $A_{2A}$ AR and $A_{2B}$ AR in NHLFs. When NHLFs were treated with TGFβ for 24 h, the expression levels of $A_{2A}$ AR (Fig. 4c, left panel) and $A_{2B}$ AR (Fig. 1a, right panel) were significantly decreased compared to those in the control. We
then examined whether alterations in the expression levels of ARs occurred in murine models of fibrotic diseases (Fig. 1b-e). Protein levels of $A_{2A}$ AR were decreased in liver tissue samples from TAA-induced liver fibrosis mice compared to the control (Fig. 1b), and protein levels of $A_{2B}$ AR were decreased in liver tissue samples from TAA-induced liver disease mice or lung tissue from BLM-induced lung fibrosis mice compared to the control (Fig. 1c). A decrease in $A_{2B}$ AR levels was also observed in liver tissue from CDAHFD-induced liver disease mice (Fig. 4a, middle panel). In contrast, the protein levels of $A_1$ AR and $A_3$ AR were not reduced in murine models of fibrotic diseases (Fig. 1d and e). $A_1$ AR levels were significantly elevated in liver tissues from TAA-induced liver disease and lung tissues from BLM-induced lung disease compared to those in the control (Fig. 1d). $A_3$ AR levels were significantly elevated in liver tissues from TAA-induced liver disease mice (Fig. 1e, left panel) and were not altered in lung tissues from BLM-induced lung disease mice (Fig. 1e, right panel). As ARs are coupled to ADCYs via G proteins, and thereby affect cAMP levels, we examined the mRNA levels of ADCYs. Messenger RNA levels of ADCYs were reduced in the TGFβ-treated NHLFs (Fig. 1f) and in lung tissue from BLM-induced lung fibrosis (Suppl. Figure 1) compared to the controls.

Cyclic AMP signaling is reduced in hepatic and pulmonary fibrotic diseases.

Reduction in the expression levels of $A_{2A}$ AR, $A_{2B}$ AR, and ADCYs suggests that cAMP signaling is attenuated in fibrotic diseases. Thus, we compared Epac levels in liver tissue from murine models of fibrotic liver diseases and found that Epac1 levels were significantly decreased in the liver tissue from mouse models of TAA-induced and CDAHFD-induced liver fibrosis compared to the control (Fig. 2A). Since we have shown that TGFβ reduces the protein levels of Epac1 in human-origin HSCs [12], we wished to examine whether TGFβ reduces the protein levels of Epac2 (Fig. 2b). Epac2 levels were found to be significantly reduced in TGFβ-treated NHLFs compared to the control. We then compared the expression levels of Epacs, Col1, and α-SMA in NHLFs and DHLFs (Fig. 2c). Compared to NHLFs, Epac1 and Epac2 levels were significantly reduced, and the levels of Col1 and α-SMA were significantly elevated in DHLFs. Elevated levels of α-SMA and Col1 in DHLFs suggest that DHLFs are activated fibroblasts. MF [12, 14, 23] or the pan AR activator NECA elevates cAMP levels, thereby activating Epac. We examined whether Epac activation using MF-S or NECA inhibited the fibrotic process in DHLFs and NHLFs. MF-S reduced Col1 levels in DHLFs in a concentration-dependent manner (Fig. 2d). In addition, NHLFs were treated with TGFβ or TGFβ + NECA for 24 h. The levels of Col and α-SMA were significantly elevated by TGFβ, while NECA treatment significantly reduced these levels (Fig. 2d and e).

These results suggest that cAMP signaling is reduced in murine models of fibrotic disease and that fibrotic progress is inhibited via activation of the cAMP/Epac pathway.

**Therapeutic treatment with MF inhibits fibrotic progress in murine models**

Prophylactic treatment with MF inhibited inflammatory and fibrotic responses in liver tissues from murine models of TAA-induced liver diseases, and MF-S may be a better alternative to (R)-isomer of MF (MF-R)
as an anti-inflammatory and anti-fibrotic agent [12]. Thus, we examined whether MF-S as a therapeutic agent inhibits inflammatory responses in murine models of TAA-induced liver fibrosis and BLM-induced pulmonary fibrosis. Compared to the control, mRNA levels of CCL2, tumor necrosis factor-α (TNFα), and interleukin-1α (IL1α) were markedly elevated in liver or lung tissue samples from mice with TAA-induced liver disease or BLM-induced pulmonary disease, respectively, and significantly reduced in mice treated with MF-S (Fig. 3a). Matrix metalloproteinase-9 (MMP9) activity was significantly enhanced in liver tissue from mice with TAA-induced liver disease; however, activity was significantly reduced in a dose-dependent manner after treating with MF-S (Fig. 3b). In addition, compared with those from the control group, protein levels of TIMP2 were significantly elevated in liver and lung tissues from mice with TAA-induced liver disease and BLM-induced lung disease, respectively, but were significantly reduced after treatment with MF-S (Fig. 3c). H&E staining of lung tissue sections was performed to observe histopathological changes occurring in the murine models. Compared with the control, cell infiltration was observed in the lung tissue of mice with BLM-induced pulmonary disease (Fig. 3d, upper panels). When BLM-induced pulmonary disease mice were treated with MF-S, cell infiltration was markedly reduced. We then examined CD45 levels in lung tissue collected from lung disease murine models (Fig. 3d, lower panels). CD45 is expressed in almost all immune cells, such as T cells, B cells, and macrophages, to confirm whether the infiltrated cells are immune cells. CD45-positive cells were not found in the tissue samples from the control mice, whereas they were found in the whole lung tissue from mice with BLM-induced lung disease. When mice with lung disease were treated with MF-S, CD45-positive cells were found in parts of the lung tissue. These results suggest that the inflammatory response activated in mice with fibrotic diseases in the liver or lung was suppressed by the MF treatment.

We then examined whether MF-S as a therapeutic intervention inhibits fibrotic responses in murine models of TAA-induced liver fibrosis and BLM-induced pulmonary fibrosis. Hydroxyproline levels in the mice liver tissue increased after TAA treatment. After a 16-week TAA treatment, hydroxyproline levels were significantly higher than those after an 8-week treatment (Fig. 4a), which suggests that the 16-week TAA treatment worsened liver fibrosis compared to the 8-week treatment. Tissue levels of hydroxyproline and serum levels of the Col1 metabolites P1NP increased in TAA-induced liver disease and BLM-induced pulmonary disease, and MF-S reduced the levels of hydroxyproline and P1NP in a dose-dependent manner (Fig. 4b). Protein levels of Col1α (Fig. 4c) and α-SMA (Fig. 4d) were also elevated in liver and lung tissues from murine models of TAA-induced liver fibrosis and BLM-induced pulmonary fibrosis, respectively; MF-S treatment reduced Col1α and α-SMA levels in murine models. We then performed immunostaining of tissue samples from murine models (Fig. 4e). Col1α was stained blue with Masson's trichrome or red with Sinus red, and reticulin and α-SMA were stained brown. Compared with the control group, the intensity of the blue or red color of Colα and brown color of reticulin and α-SMA were markedly higher in tissues from mice with TAA-induced liver disease and BLM-induced lung disease. Col deposition occurred around the periportal area and between the portal veins in mice with TAA-induced liver disease. When mice with fibrotic diseases were treated with MF, the Colα and α-SMA levels were markedly reduced. These results suggest that the fibrotic processes that cause the progression of liver and lung diseases in murine models were suppressed by MF treatment.
MF activates cAMP signaling via upregulating A\textsubscript{2A} AR and A\textsubscript{2B} AR

Cyclic AMP signaling by MF was blocked by pharmacological inhibition of A\textsubscript{2A} AR and A\textsubscript{2B} AR, suggesting that A\textsubscript{2A} AR and A\textsubscript{2B} AR are involved in the effects of MF [23]. Thus, we examined whether MF-S treatment affects the expression levels of A\textsubscript{2A} AR and A\textsubscript{2B} AR in lung and liver tissue from murine models of fibrotic diseases and in TGF\beta-treated NHLFs. MF-S treatment restored the protein levels of A\textsubscript{2A} AR in TAA-induced liver fibrosis (Fig. 5a) and A\textsubscript{2B} AR in the mouse models of CDAHFD-induced liver fibrosis and BLM-induced lung fibrosis (Fig. 5b). In addition, when NHLFs were treated with TGF\beta over 24 h, the protein levels of A\textsubscript{2A} AR (Fig. 5c, left panel) and A\textsubscript{2B} AR (Fig. 1b, right panel) were reduced. This TGF\beta-induced reduction in the levels of A\textsubscript{2A} AR and A\textsubscript{2B} AR was observed to be blocked by MF-S (Fig. 5c).

We then examined ADCYs mRNA levels in murine models of BLM-induced pulmonary fibrosis. The mRNA levels of ADCYs were reduced in BLM-induced lung fibrosis compared to the control, and MF-S blocked the reduction in ADCYs (Supplementary Fig. 1). Since these results suggest that cAMP signaling in murine models of fibrotic diseases is restored after treatment with MF-S, we examined Epac1 levels in murine models of fibrotic diseases. Epac1 levels were reduced in liver and lung tissues from murine models of fibrous diseases compared to the control and were significantly elevated in those from MF-S-treated murine models (Fig. 5d). Since an Epac-mediated pathway modulated the expression levels of K\textsubscript{Ca}2.3 and K\textsubscript{Ca}3.1, in murine models of liver fibrosis and HSCs [12], we examined whether similar changes in K\textsubscript{Ca}2.3 and K\textsubscript{Ca}3.1 levels occurred in lung tissue from murine models of pulmonary fibrosis and TGF\beta-treated NHLFs. K\textsubscript{Ca}3.1 levels were elevated in lung tissue from murine models of pulmonary fibrosis compared to the control and were significantly reduced in samples from MF-S-treated fibrosis models compared to those from BLM-induced lung fibrosis (Fig. 5e, left panel). In addition, K\textsubscript{Ca}2.3 levels were elevated by TGF\beta in NHLFs; elevated K\textsubscript{Ca}2.3 was then reversed by MF-S in a concentration-dependent manner, or by NECA (Fig. 5e, middle and right panels). These results suggest that A\textsubscript{2A} AR and A\textsubscript{2B} AR upregulation inhibits the fibrotic process by activating the cAMP pathway.

Discussion

This is the first study to demonstrate that downregulation of A\textsubscript{2A} AR and A\textsubscript{2B} AR contributes to the development of fibrotic diseases, and that fibrotic progression can be inhibited by upregulating A\textsubscript{2A} AR and A\textsubscript{2B} AR. Downregulation of A\textsubscript{2A} AR and A\textsubscript{2B} AR and a reduction in Epac levels were observed in murine models of fibrotic liver and lung diseases, suggesting that downregulation of A\textsubscript{2A} AR and A\textsubscript{2B} AR contribute to the development of fibrotic diseases in the liver and lung by attenuating cAMP signaling. MF treatment upregulated A\textsubscript{2A} AR and A\textsubscript{2B} AR, elevated Epac levels, and inhibited inflammatory and fibrotic responses in murine models of fibrotic diseases, suggesting that the upregulation of A\textsubscript{2A} AR and A\textsubscript{2B} AR inhibits inflammatory and fibrotic processes by enhancing cAMP signaling. Therefore, A\textsubscript{2A} AR and A\textsubscript{2B} AR may be therapeutic targets against inflammatory and fibrotic diseases, including liver and lung fibrosis,
and A2A AR and A2B AR-upregulating agents, such as MF, are potential therapeutic agents for fibrotic diseases.

Cyclic cAMP modulates inflammatory and fibrotic responses via Epac- and PKA-mediated pathways. Cyclic AMP/Epac signaling exerts anti-fibrotic actions by inhibiting epithelial–mesenchymal transformation and ECM formation. In contrast, pro-fibrotic agents, such as TGFβ and angiotensin II, downregulate Epac, thereby promoting fibrotic responses [9]. In addition, cAMP/PKA signaling can inhibit inflammatory and fibrotic responses. Continuously elevated cAMP levels inhibit immune cell functions, such as the proliferation and activation of T and B cells [8], and PKA signaling may be involved in anti-inflammatory and anti-fibrotic pathways [8, 24]. Downregulation of K\textsubscript{Ca}2.3 and K\textsubscript{Ca}3.1 may play a critical role in the anti-inflammatory and anti-fibrotic effects of cAMP. Pro-inflammatory cytokines and pro-fibrotic growth factors, such as TGFβ [12] and VEGF [25], upregulate K\textsubscript{Ca}3.1 and K\textsubscript{Ca}2.3, and pharmacological inhibition or knockdown of K\textsubscript{Ca}3.1 and K\textsubscript{Ca}2.3 inhibits in vitro and in vivo fibrotic responses [12, 13, 26, 27]. Cyclic AMP was found to reduce the expression of K\textsubscript{Ca}3.1 and K\textsubscript{Ca}2.3 via an Epac-mediated pathway [12], and inhibited the K\textsubscript{Ca}3.1 current via PKA-dependent phosphorylation of K\textsubscript{Ca}3.1 [14]. MF may exert anti-fibrotic and anti-fibrotic effects via cAMP/Epac and PKA/K\textsubscript{Ca}2.3 and K\textsubscript{Ca}3.1-mediated pathways in various organs, such as the liver and lungs [12].

A2A AR and A2B AR activate cAMP signaling via the G\textsubscript{s} protein, whereas A1 AR and A3 AR inhibit cAMP signaling via the G\textsubscript{i} protein. Thus, the downregulation of A2A AR and A2B AR and upregulation of A1 AR and A3 AR, as observed in murine models of fibrotic diseases, indicate that cAMP signaling is suppressed. Suppression of cAMP signaling may contribute to the development of fibrotic diseases. In contrast, upregulation of A2A AR and A2B AR and downregulation of A1 AR and A3 AR, as observed in MF-treated murine models of fibrotic diseases, indicate that cAMP signaling is augmented by MF treatment in fibrotic models. Therefore, augmented cAMP signaling may attenuate inflammatory and fibrotic progression in fibrotic diseases. These results suggest that A2A AR and A2B AR play important roles in the inhibition of liver and pulmonary fibrosis.

A2A AR is distributed in the liver, lungs, and immune system and is expressed in immune cells and fibroblasts [16]. The A2A AR acts as an endogenous modulator of inflammation and tissue repair. Expression levels of A2A AR were markedly decreased in lung tissue from a murine model of BLM-induced pulmonary fibrosis (Fig. 1), which is consistent with the finding that A2A AR was downregulated in patients with severe IPF [28]. In addition, A2A AR-null mice were more susceptible to BLM-induced lung injuries [17]. These results suggest that A2A AR is a potential target for inflammatory and fibrotic diseases.

The role of A2B AR in the inflammatory response remains controversial. A2B AR is distributed in the bowels and lungs and expressed on immune cells [16]; in addition, A2B AR has both pro-inflammatory and anti-inflammatory effects. Pharmacological inhibition or knockout of A2B AR suppresses intestinal
inflammation in murine colitis models [29] and exacerbates inflammation of dextran sodium sulfate colitis in mice [30]. Anti-inflammatory effects are generated by coupling with protein G_s, while proinflammatory effects are generated by coupling with protein G_q [19]. However, A_{2B} AR may be a potential target for treating acute lung injuries [31]. Pharmacological inhibition or deletion of the A_{2B} AR enhanced pulmonary inflammation, and an A_{2B} AR agonist attenuated pulmonary inflammation.

It is unclear how MF restores downregulated A_{2A} AR and A_{2B} AR in murine models of fibrotic diseases and TGFβ-treated NHLFs, and how MF blocks TGFβ-induced alterations in K_{Ca2.3}, K_{Ca3.1}, Col1, and α-SMA. It is known that MF increases cAMP levels in various cells, and cAMP regulates the gene expression of various molecules. As shown by the cAMP regulation of K_{Ca2.3} and K_{Ca3.1} expression, MF may regulate the expression levels of these proteins by enhancing cAMP signaling. Cyclic AMP-induced K_{Ca2.3} and K_{Ca3.1} downregulation may inhibit Col1 and α-SMA production by attenuating Ca^{2+} signaling. However, further studies are necessary to clarify the mechanism by which MF regulates AR expression.

Chronic persistent inflammation and epithelial mesenchymal transition (EMT) are two important pathogenic events of fibrotic diseases, including IPF [32]. Therefore, the inhibition of inflammation or EMT may be an efficient therapeutic strategy for fibrotic diseases. As observed in the present study, downregulation of A_{2A} AR and A_{2B} AR contributed to the development of fibrotic diseases in the liver and lungs, whereas upregulation of A_{2A} AR and A_{2B} AR inhibited inflammatory and fibrotic responses. The activation of inflammatory and fibrotic processes, as evidenced by reduced Epac signaling and α-SMA upregulation, was inhibited by the upregulation of A_{2A} AR and A_{2B} AR. These results strongly suggest that A_{2A} AR- and A_{2B} AR-upregulating agents, such as MF, may serve as novel therapies to inhibit inflammatory and fibrotic progression via cAMP-mediated pathways.

**List Of Abbreviations**

- **α-SMA** α-smooth muscle actin
- **ADCY** adenylyl cyclase
- **Bleomycin** BLM
- **cAMP** cyclic adenosine monophosphate
- **CDAHFD** choline-deficient, L-amino-acid-defined, high-fat diet with 0.1% methionine
- **Col** collagen
- **DMSO** dimethyl sulfoxide
- **Epac** exchange protein directly activated by the cAMP
HSC hepatic stellate cell
H&E hematoxylin and eosin
IL interleukin
IPF idiopathic pulmonary fibrosis
MF modafinil
MF-S (S)-isomer of modafinil
NECA 1-(6-Amino-9H-purin-9-yl)-1-deoxy-N-ethyl-β-D-ribofuranuronamide
PCR polymerase chain reaction
P1NP procollagen type1 N-terminal propeptide
TAA thioacetamide
TIMP tissue inhibitor of metalloproteinases
TGFβ transforming growth factor-β
TNFa tumor necrosis factor-α

Declarations

Ethical Approval  Experiments involving mice were approved by the Institutional Review Board for Human Research and Animal Care and Use Committee of the Ewha Womans University, Seoul, South Korea (EUM20-052). Experiments were conducted in accordance with the Declaration of Helsinki, Animal Care Guidelines of the Ewha Womans University Medical School, and Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised in 1996).

Competing interests  The authors declare that they have no conflict of interest.

Authors' contributions  Haiyan Li, Jee Aee Kim, Seong-Eun Jo, Huisu Lee: performed the experiments; Haiyan Li and Suk Hyo Suh: wrote the manuscript; Kwan-Chang Kim, Shinkyu Choi, and Suk Hyo Suh: conceived and designed the research, supervised project, edited manuscript, obtained funding.

Funding  This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NRF-2022R1A2C1007823), NRF grant funded by the Korea government (MSIT) (NRF-2022R1A2C1092484), Ewha Womans University Research Grant of 2022 (1-2022-1108-001-1) and intramural research promotion grants from Ewha Womans University, School of Medicine (RP-2020).
Availability of data and materials  The datasets and materials generated and analysed during the current
study are available from the corresponding authors on reasonable request.

References

   (7835):555-566. https://doi.org/10.1038/s41586-020-2938-9

   https://doi.org/10.3389/fphys.2017.00777

3. Hecker L, Logsdon NJ, Kurundkar D, Kurundkar A, Bernard K, Hock T, Meldrum E, Sanders YY,
   Thannickal VJ (2014) Reversal of persistent fibrosis in aging by targeting Nox4-Nrf2 redox
   imbalance. Sci Transl Med 6 (231):231ra47. https://doi.org/10.1126/scitranslmed.3008182


   https://doi.org/10.1186/s12931-021-01791-z

   Disease: Epidemiology, Natural History, and Diagnostic Challenges. Hepatology 64 (3):954.
   https://doi.org/10.1002/hep.28719

8. Raker VK, Becker C, Steinbrink K (2016) The cAMP Pathway as Therapeutic Target in Autoimmune

   https://doi.org/10.1111/j.1476-5381.2012.01847.x

    Protein kinase a mediated anti-inflammatory effects exerted by adenosine treatment in mouse

    smooth muscle cell proliferation. J Mol Cell Cardiol 50 (1):87-98.
    https://doi.org/10.1016/j.yjmcc.2010.10.010


Figures
Figure 1

Expression levels of ARs and ADCYs are altered in fibrotic diseases

Protein levels of ARs (A-E) and mRNA levels of ADCYs (F) were measured by western blotting and RT-PCR, respectively. (A) Expression levels of A2A AR and A2B AR in NHLFs, DHLFs, and TGFβ-treated NHLFs. The blots shown are representative of three to five experiments performed using three to five different cultures. (B) Expression levels of A2A AR in liver tissues from wild-type and murine models of TAA-
induced liver fibrosis. (C) Expression levels of A2B AR in liver and lung tissues from wild-type and murine models of TAA-induced liver fibrosis or BLM-induced lung fibrosis. (D and E) Expression levels of A1 AR (D) and A3 AR (E) in the liver and lung tissues from wild-type and murine models of TAA-induced liver fibrosis or BLM-induced lung fibrosis, respectively. (F) Messenger RNA levels of ADCYs in NHLFs and TGFβ-treated NHLFs. NHLFs were treated with TGFβ for 24 h. Data are presented as the mean ± SEM values. **P < 0.01 vs. NHLFs or control.
Figure 2

cAMP signaling is reduced in fibrotic diseases

(A) Expression levels of Epac1 in liver tissues from wild-type and murine models of liver fibrosis. Epac1 was significantly downregulated in liver fibrosis models compared to the control. (B) Expression levels of Epac2 in NHLFs. Treatment with TGFβ (10 ng/ml) for 24 h reduced Epac2 levels. (C) Expression levels of Epac1, Epac2, α-SMA, and Col1 in NHLFs and DHLFs. Epac1 and Epac2 levels were significantly reduced, and α-SMA and Col1 levels were significantly elevated in DHLFs compared to the NHLFs. (D and E) Expression levels of Col1 (D) and α-SMA (E) in NHLFs. TGFβ(10 ng/ml) augmented expression levels of Col1 and α-SMA. Augmented levels of Col1 and α-SMA were reversed using cAMP-elevating agents: NECA (100 nM) or MF-S. (B-E) Blots shown are representative of the three to five experiments performed using three to five different cultures. Data are presented as mean ± SEM values. **P < 0.01 vs. control. #P < 0.05, ##P < 0.01 vs. DHLFs or TGFβ-treated NHLFs.
Figure 3

MF-S inhibits inflammatory responses in fibrotic diseases

(A) Messenger RNA levels of CCL2, TNF-a, and IL1bin liver or lung tissues from murine models of TAA-induced liver fibrosis or BLM-induced lung fibrosis. (B) MMP9 activity in liver tissues from murine models of TAA-induced liver fibrosis. (C) Protein levels of Timp2 in liver or lung tissues from murine models of TAA-induced liver fibrosis. (D) H&E and CD45 staining in liver tissues from murine models of TAA-induced liver fibrosis.
TAA-induced liver fibrosis or BLM-induced lung fibrosis. (D) Representative images of H&E and CD45 staining in lung tissues from mice with BLM-induced pulmonary fibrosis. Enlargements of black boxes (solid line) were shown in right panels, respectively. Data are presented as mean ± SEM values. **$P < 0.01$ vs. control. #P$<0.05$, ##$P < 0.01$ vs. BLM-induced lung disease model.
MF-S inhibits fibrotic responses in murine models of fibrotic diseases

(A and B) Hydroxyproline or P1NP levels in murine models of TAA-induced liver fibrosis or BLM-induced lung fibrosis. (A) Hydroxyproline levels in liver tissue from mice treated with TAA for 8 or 16 weeks. (B) Hydroxyproline or P1NP levels were elevated in models of fibrotic diseases, and elevated levels of hydroxyproline or P1NP were significantly reversed after MF-S treatment. (C and D) Expression levels of Col1 (C) or α-SMA (D) in TAA-induced liver fibrosis and BLM-induced lung fibrosis murine models. (E) Representative images of Masson’s trichrome, Sirus red, reticulin, and α-SMA staining in lung tissues from mice with BLM-induced pulmonary fibrosis. Enlargements of black boxes (solid line) were shown in right panels. Data are presented as mean ± SEM values. **P < 0.01 vs. control. #P<0.05, ##P < 0.01 vs. BLM-induced lung disease model.
Figure 5

MF-S augments cAMP signaling by upregulating $A_{2A}$ AR and $A_{2B}$ AR

(A and B) Reduced $A_{2A}$ AR (A) and $A_{2B}$ AR (B) levels in murine models of fibrotic diseases were restored after treatment with MF-S. (C) NHFs were treated with TGFb or TGFb+MF over 24 h. TGFb reduced expression levels of $A_{2A}$ AR (C) and $A_{2B}$ AR (Fig. 1C). The $A_{2A}$ AR and $A_{2B}$ AR levels were restored after...
MF-S treatment. (D) Downregulated Epac1 were restored by treatment with MF in murine models of fibrotic diseases. (E) Expression levels of $K_{Ca}3.1$ in liver tissues from BLM-induced lung fibrosis (left panel) or $K_{Ca}2.3$ in NHLFs (middle and right panels). TGFβ elevated $K_{Ca}2.3$ levels, and this elevation was reversed by MF or NECA. (C-E) Blots shown are representative of the three to five experiments performed with three to five different cultures. Data are presented as mean ± SEM values. **$P < 0.01$ vs. control. $P < 0.05$, ##$P < 0.01$ vs. TAA-induced liver disease model or BLM-induced lung disease model.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SF1.png