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ORIGINAL RESEARCH

Interferon-gamma inhibits adenosine A2A receptor function in hepatic stellate cells by STAT1-mediated repression of adenylyl cyclase

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Background and purpose: Adenosine, an endogenous purine nucleoside, is a potent regulator of the inflammatory response and stimulus for fibrosis. We have previously demonstrated that adenosine, acting at the A2A receptor, plays a central role in hepatic fibrosis via direct promotion of collagen production by hepatic stellate cells. As we have previously demonstrated that macrophage A2A receptor function is regulated by interferon-gamma (IFNγ), a noted anti-fibrotic but pro-inflammatory cytokine, we examined its effect on A2AR-stimulated collagen production in the human hepatic stellate cell line LX-2.
Experimental approach: Collagen expression was determined by western blotting and realtime reverse transcription polymerase chain reaction (RT-PCR). Receptor desensitization was assessed by western blotting for membrane associated GRK2. Receptor signaling was determined by western blotting for phosphorylated extracellular signal-related protein kinase (ERK) protein and immunoassay for intracellular cyclic AMP (cAMP). siRNA was used to knock down expression of adenylyl cyclase and signal transducer and activator of transcription (STAT). Adenylyl cyclase expression was assessed by realtime RT-PCR, and STAT expression was assessed by western blotting.
Key results: IFNγ diminishes A2A receptor-mediated collagen production at both protein and mRNA levels. IFNγ alters signal transduction at A2A receptors by a STAT1 mediated mechanism involving the suppression of adenylyl cyclase expression.
Conclusions and implications: IFNγ inhibits the function of the adenosine A2A receptor in hepatic stellate cells by downregulating the expression of adenylyl cyclase. This finding explains, at least in part, the protective effect of IFNγ in hepatic fibrosis.
Keywords: hepatic fibrosis, collagen-1, interferon-gamma, inflammation, adenylyl cyclase, siRNA, hepatic stellate cells

Introduction

Adenosine is a purine nucleoside released during times of cellular stress.[1] Adenine nucleotide release is the major source of extracellular adenosine,[2] which regulates inflammation,[3,4] cholesterol metabolism,[5] wound healing[6] and fibrosis,[7] etc, via interaction with cell surface receptors. There are 4 known mammalian adenosine receptors: A1, A2A, A2B, and A3, all of which belong to the G-protein coupled receptor (GPCR) family.[8,9] Expression of these receptors varies among cell types and is regulated by multiple factors.[1] External factors,[10] receptor localization,[11] and sensitization[12] all play a role in the functional regulation of adenosine receptors.

Previous work by our laboratory and others has shown that the inflammatory cytokines interferon-gamma (IFNγ), tumor necrosis factor alpha (TNFα), and interleukin-1

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(IL-1) regulate A2A receptor expression and function in monocytes.[13] IFNγ is a negative regulator of function; while TNFα and IL-1 increase A2A receptor expression and enhance function by impairing desensitization of the A2A receptor.[14] TNFα and IL-1 block desensitization by preventing G-protein coupled receptor kinase 2 (GRK2) and β-arrestin association with the membrane in a sphingomyelinase-dependent manner.[14] The mechanism by which IFNγ inhibits A2A receptor function remains unknown. It is also undetermined whether or not IFNγ inhibits A2A receptor function in other cell types besides monocytes.

Adenosine plays an important role in the development of multiple forms of fibrosis, including hepatic fibrosis. Adenosine A2A receptor (A2AR) expression is upregulated in liver homogenates from mice and humans with hepatic fibrosis, and protection from fibrosis has been observed in A2A receptor knockout mice.[15,16] Adenosine also regulates chemotaxis in hepatic stellate cells,[17] and recent work in our laboratory has demonstrated that activation of the A2A receptor provides a pro-fibrotic signal to these cells.[18] Furthermore, we have observed that adenosine, generated extracellularly from adenine nucleotides, plays a critical role in the pathogensis of hepatic fibrosis in vivo.[17]

Therefore, we examined the effect of IFNγ on A2A receptor function in LX-2 cells, a human hepatic stellate cell line.[19] We observed that IFNγ has the same negative effect on adenosine receptor signaling and function, particularly collagen production, in human hepatic stellate cells as it does in monocytes.[13] We further demonstrated that IFNγ downregulates adenylyl cyclase expression, which is required for A2A receptor-mediated regulation of collagen production. We conclude that IFNγ inhibits the function of the A2AR in hepatic stellate cells by downregulating the expression of adenylyl cyclase.

Materials and methods

Materials

Materials were as follows: cAMP Biotrak Enzymeimmunoassay System (GE Healthcare, Amersham UK), GRK2 Antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Beta-Actin Antibody (Abcam), Anti-Type 1 Collagen Antibody (Southern Biotech, Birmingham, AL, USA), anti-phospho-ERK1 & 2 (extracellular signal-related protein kinases 1 and 2) Antibody (Biosource – now Invitrogen, Carlsbad, CA, USA), Anti-ERK1 & 2 pan Antibody (Biosource), recombinant human IFNγ (R&D Systems, Minneapolis, MN, USA), Recombinant Human TNFα (R & D Systems), recombinant human tumor growth factor beta 1 (TGF-β1) (R & D Systems), CGS 21680 Hydrochloride (Tocris Bioscience, Ellisville, MO, USA), Stellate Cell Growth Supplement (ScienCell Research Laboratories, San Diego, CA, USA), 10× Cell Lysis Buffer (Cell Signaling, Beverly, MA, USA), Brilliant SYBR Green QPCR Master Mix (Stratagene), BCA Assay (Pierce, Rockford IL, USA), Mission Transduction Particles NM_003150, NM_007315, NM_004036, NM_001114, and NM_001116 (Sigma-Aldrich, St. Louis, MO, USA). The LX-2 human hepatic stellate cell line was characterized and provided by Dr SL Friedman from Mount Sinai Medical School, NY, USA.[19]

Cell culture

LX-2 human hepatic stellate cells were grown in T75 size tissue culture flasks in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine at 37°C in a humidified atmosphere containing 5% CO_2. Trypsin was used to re-plate cells into T25 flasks or 6-well or 24-well tissue culture plates for experiments. Experiments requiring serum-free media were conducted using DMEM supplemented with 1% penicillin/streptomycin, 1% L-glutamine, and 1% stellate cell growth serum (SteCGS) instead of 10% fetal bovine serum.

RNA interference

Viral particles expressing siRNA sequences against AC3, AC9, STAT1, and STAT3 were purchased commercially from Sigma-Aldrich (see Materials). Viral particles were independently verified for efficacy and specificity by Sigma-Aldrich. 3×10^4 LX-2 cells were plated into 6-well tissue culture plates. 24 hours later, cells were treated with 8 μg hexadimethrine bromide per mL of medium. Viral particles were added immediately at suitable multiplicities of infection (MOI) according to manufacturer's instructions. The following day, medium containing viral particles was removed and cells were washed 1× with phospho-buffered saline (PBS) and incubated in fresh cDMEM. 24 hours later, medium was replaced with cDMEM containing 10 μg/mL puromycin for selection of transduced cells. Cells were grown in puromycin containing medium (replaced every 3–4 days) for 10 days to 2 weeks until resistant colonies could be identified. Resistant colonies were expanded and used in experiments.

Protein extraction

Cells were grown in T25 flasks until 80%–90% confluent. Media was removed and cells were washed with PBS. Media was replaced with serum-free DMEM. Cytokines were added to each flask, and cells were incubated overnight for

18–24 hours. Following overnight incubation, CGS21680 (final concentration 1 μM) or 1 ng/mL TGF-β1 (final concentration 1) was added, and cells were incubated again. To measure collagen production, cells were incubated for 24 hours. To measure GRK2 translocation, cells were incubated for 10 minutes. To measure ERK1/2 phosphorylation, cells were incubated for 5 minutes. To measure STAT expression, cells were left untreated in complete DMEM in T25 flasks. Each separate experiment consisted of individual flasks receiving different combinations of cytokines and CGS21680 or TGF-β1. Controls were given DMSO or buffer instead of cytokines or CGS21680 or TGF-β1, as appropriate. Each flask resulted in a single sample. Each sample was run multiple times on SDS-PAGE gels and results were averaged to give a result for that experiment. N values in the data reflect repetition of each separate experiment.

To prepare whole cell lysates, cells were washed once with PBS and incubated on a rocker for 1 hour with 200–300 μL cell lysis buffer plus protease inhibitors per T25 flask at 4°C. After the incubation, plates were scraped and the lysates transferred to a microcentrifuge tube on ice. Tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatants containing whole cell extract were transferred to a clean microcentrifuge tube on ice. Pellets were discarded. Protein concentration in extracts was determined by a standard BCA assay according to the manufacturer's instructions. Samples were then run on an SDS-PAGE gel or frozen at –20°C for later use.

To prepare crude membrane fractions, cells were washed once with PBS and incubated with trypsin for 5 minutes. Cells were transferred to microcentrifuge tubes and pelleted by a 30 second pulse in a microcentrifuge, then washed once with PBS and pelleted again. Cells were resuspended in 500 μL ice-cold PBS with protease inhibitor and left on ice. Each tube was sonicated 3 times at maximum level for 8–10 seconds each time. Cells were left on ice for 15–20 seconds in between each sonication. After sonication, tubes were centrifuged at 300 × *g* for 10 minutes at 4°C. Supernatant was transferred to another ice-cold tube, and the remaining pellets were discarded. To isolate membrane protein, the tubes were centrifuged at maximum speed for 30 minutes at 4°C. Supernatant containing the cytosolic fraction of the cells was removed and discarded, or saved by freezing at −80°C. The pellet was resuspended in 100 μL ice-cold PBS plus protease inhibitors and stored at −80°C. Protein concentrations were determined by a standard BCA assay according to the manufacturer's instructions. Samples were then run on an SDS-PAGE gel or frozen at –20°C for later use.

Western blotting

Equal amounts of protein (8–40 μg/lane) were separated on a 7.5% or 10% SDS-PAGE gel as appropriate and transferred to nitrocellulose membranes. Membranes were stained with Ponceau to confirm effective transfer, then blocked for 2 hours rocking at room temperature in tris-buffered saline (TBST) containing either 3% bovine serum albumin (for phospho-proteins) or 5% dry milk. Blots were then incubated overnight rocking at 4°C with primary antibody diluted in blocking buffer to the manufacturer's recommended concentration. Blots were washed 3–4 times with TBST and incubated for 2 hours rocking at room temperature with alkaline phosphatase or horseradish peroxidase conjugated secondary antibody. Blots were again washed 3–4 times with TBST and exposed for 5 minutes to either enhanced chemifluorescence (ECF) or enhanced chemiluminescence (ECL) substrate. ECF exposed blots were scanned using the Storm 860 Phosphoimager, while ECL exposed blots were imaged using the Gel Logic 2200 Imaging System. Band intensity was quantitated using Molecular Imaging or ImageQuant software. To screen for another protein, blots were stripped after imaging and re-probed.

RNA extraction

Cells were grown in T25 flasks until 80%–90% confluent. Media was removed and cells were washed with PBS. Media was replaced with serum-free DMEM. IFNγ was added to each flask, and cells were incubated for 4, 12, or 24 hours. Control cells were not incubated with IFNγ. Each separate experiment consisted of individual flasks receiving IFNγ (or buffer as control) for a different length of time. Each flask resulted in a single sample. Each sample was analyzed by realtime reverse transcription polymerase chain reaction (RT-PCR) in duplicate or triplicate and results were averaged to give a result for that experiment. N values in the data reflect repetition of each separate experiment.

To isolate total cellular RNA, cells were washed once with PBS and incubated in 2 mL Trizol per T25 flask. Trizol was added directly to the flasks, and flasks were incubated at 37°C for 5 minutes. Supernatant was transferred to microcentrifuge tubes (2 tubes per plate, 1 mL per tube) and frozen at −80°C or used immediately. If frozen, samples were thawed and incubated for at least 5 minutes at room temperature to dissociate of nucleoprotein complexes. 200 μL of chloroform was added to each tube, tubes were vortexed briefly and incubated at room temperature for 3 minutes, and were then centrifuged at 12,000 × *g* for 15 minutes at 4°C. Following centrifugation, the mixture was separated into a lower, red organic phase and an upper, clear, aqueous phase. RNA remained in the aqueous phase. The upper, clear, aqueous phase was transferred by pipet to a new tube. 500 μL of isopropanol was added to the tube, tubes were vortexed and incubated at room temperature for 10 minutes, and were then centrifuged at 12,000 × *g* for 15 minutes at 4°C. Following centrifugation, the RNA formed a gel-like pellet on the side/bottom of the tube. Supernatant was removed and pellet washed with 1 mL of 75% ethanol by vortexing briefly and centrifuging at no more than 7,500 × *g* for 5 minutes at 4°C. Supernatant was again removed, and pellet air dried. RNA was redissolved in 8 μL of RNase-free water by pipetting, like tubes were combined, and RNA was incubated for 10 minutes at 55°C–60°C. Following incubation, RNA concentration was determined. RNA was stored at −80°C unless used immediately in reverse transcriptase reaction.

Realtime RT-PCR

cDNA was reverse transcribed from 3.0 μg mRNA in a 50 μL reaction containing $MgCl_2$, 10X buffer, dNTPs, RNase inhibitor, reverse transcriptase, and a poly(t) primer to ensure only mRNA was transcribed. The reaction occurred over 1 hour at 45°C. The resultant cDNA was amplified in a spectrofluorometric thermal cycler (Stratagene, Cedar Creek, TX, USA) using Brilliant SYBR Green QPCR Master Mix (Stratagene cat# 600548) according to the manufacturer's instructions. mRNA levels were standardized using amplification of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), whose consistency was confirmed by amplification of the housekeeping gene β-actin. PCR primer sequences for adenylyl cyclase isoforms were previously published by Kolachala et al.[20] GAPDH primer sequences are: CATCATCCCTGCCTCTAC (sense) and 5' CCTGTTGCTGTAGCCAAAT (antisense).

To quantitate expression of all 9 adenylyl cyclase isoforms, mRNA was denatured at 95°C for 15 minutes, then amplified using 45 cycles of denaturation (95°C for 30 seconds), annealing (60°C for 30 seconds), and extension (72°C for 1 minute). SYBR green fluorescence was measured at the end of each extension step, C(t) values were calculated for each curve, and relative expression levels quantitated using the following formula: fold increase = 2^[(goi control − hkg control) − (goi stimulated − hkg stimulated)], where 'goi' represents the gene of interest and 'hkg' represents the housekeeping gene GAPDH. Specificity of the final products was determined by melting curve analysis and gel electrophoresis. After amplification, a final melting curve was recorded by denaturating the products (95°C for 1 minute), cooling the PCR mixture to 55°C for 30 seconds, and then slowly heating it to 95°C at 30 seconds. SYBR green fluorescence was measured continuously during the heating step. Products were run on a 2% agarose gel stained with ethidium bromide. The size of the products on the gel matched the calculated size.

Cyclic AMP (cAMP) quantification

Cells were plated in a 24-well tissue culture plate and treated according to manufacturer's instructions for the Amersham cAMP Biotrak Enzymeimmunoassay System (GE Healthcare cat#RPN225). 24 hours after cells were plated, media was removed and cells were washed with PBS. Media was replaced with serum-free DMEM, cytokines were added to each flask, and cells were incubated overnight for 18–24 hours. Following overnight incubation, CGS21680 (final concentration 1 μM) was added, and cells were incubated for 2, 5, 10, or 20 minutes. Each separate experiment consisted of 4 replicate 24-well plates. Each well received a different combination of cytokines and CGS21680 (not all wells were used in each experiment). Controls were given DMSO or buffer instead of cytokines or CGS21680, as appropriate. All 4 replicates were run multiple times on SDS-PAGE gels, and results were averaged to give a result for that experiment. N values in the data reflect repetition of each separate experiment.

Immediately following incubation, plates were placed on ice. Supernatants were removed, and cells were washed with ice-cold PBS. Intracellular cAMP levels were determined according to the manufacturer's instructions for the Amersham cAMP Biotrak Enzymeimmunoassay System (GE Healthcare cat#RPN225), with the following changes: samples were frozen after lysis and thawed before application to the manufacturer's 96-well plate, and the final colormetric reaction was stopped after approximately 15 minutes acccording to kit instructions. cAMP concentrations were normalized to protein content of each sample by performing a BCA assay with leftover samples in the manufacturer's lysis buffer.

Statistical analysis

Statistical analysis was performed using GraphPad Prism and Statmate software (GraphPad Software, Inc., San Diego, CA, USA). Data is presented as mean ± standard error of the mean where appropriate. ANOVA and *t*-test were used to determine statistical significance; differences with a *P* value under 0.05 were considered significant.

Results

IFNγ impairs the ability of the A2AR specific agonist CGS21680 to induce collagen production in hepatic stellate cells

We have previously reported that the A2A receptor specific agonist CGS21680 induces collagen production in primary human hepatic stellate cells as well as the LX-2 human hepatic stellate cell line in a concentration-dependent manner.[18] Since IFNγ has been shown to inhibit A2A receptor function in other cell lines,[13] we examined the ability of IFNγ to inhibit A2A receptor stimulation of collagen production in LX-2 cells. CGS21680 increased collagen-1 protein production (21% ± 5% increase relative to control, $P < 0.05$, n = 7). The presence of TNFα enhanced CGS21680-mediated stimulation of collagen-1 protein production (37% ± 18% increase relative to control, $P < 0.05$, n = 5) while in the presence of IFNγ the A2A receptor agonist did not stimulate collagen-1 protein production (8% ± 4% decrease relative to control, P = n.s., n = 7) (Figure 1A).

Since both IFNγ and TNFα affect basal collagen production in LX-2 cells, we determined whether the effects of IFNγ and TNFα on A2A receptor mediated collagen production were specific by measuring the effect of IFNγ and TNFα treatment on TGF-β1 induced collagen-1 production. As expected, TGF-β1 increased collagen-1 production (44% ± 3% increase relative to control, $P < 0.05$, n = 3). The effect of TGF-β1 on collagen production was unaffected by TNFα (45% ± 21% increase relative to control, $P < 0.05$, n = 5) and IFNγ (52% ± 22% increase relative to control, $P < 0.05$, n = 3) (Figure 1B). Thus, IFNγ specifically inhibits A2A receptor mediated collagen production in LX-2 cells. Both IFNγ and TNFα impair basal collagen-1 production in LX-2 cells, consistent with previous results in the literature.[21]

IFNγ impairs the ability of CGS21680 to induce ERK phosphorylation in hepatic stellate cells

Our laboratory recently characterized the downstream signaling pathway that leads to A2A receptor mediated collagen production in LX-2 cells[18] (Figure 2A). Canonical G-protein

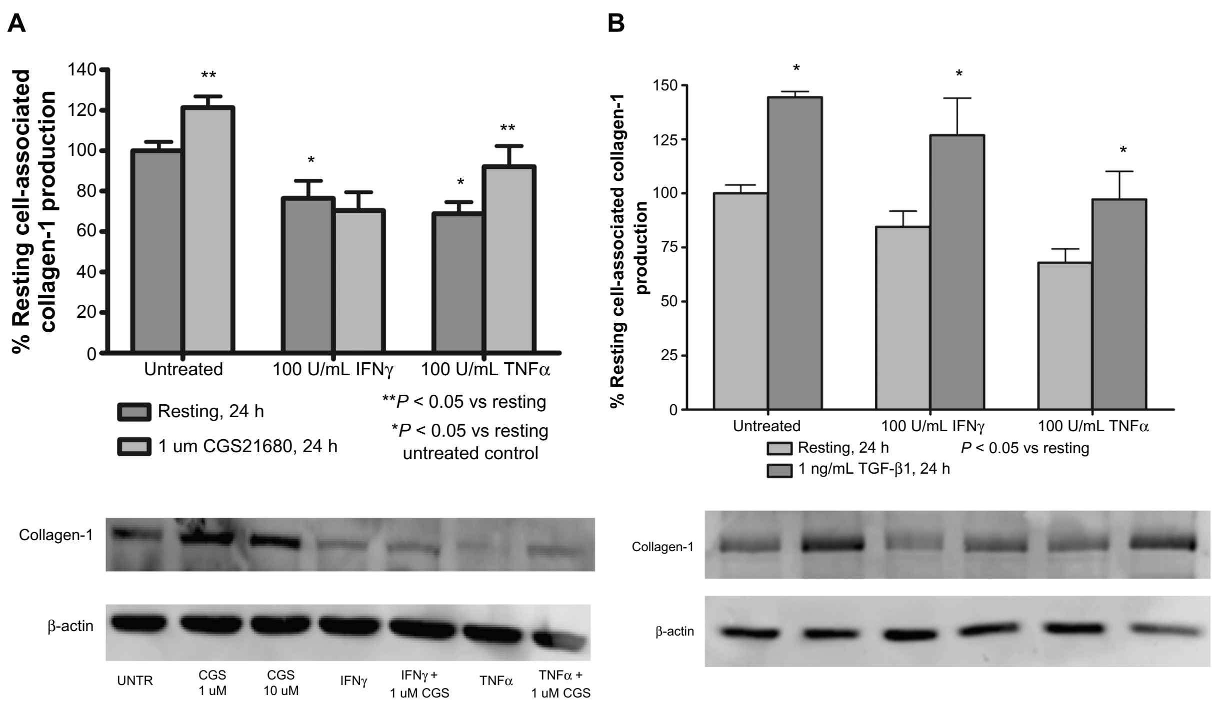

Figure 1 IFNγ inhibits A2A receptor mediated collagen 1 production in LX-2 cells. LX-2 cells were cultured overnight in the presence or absence of IFNγ and TNFα and treated with the A2AR agonist CGS21680 (A) or TGF-β1 (B) for 24 hours as described in the Materials and methods section. Cellular collagen-1 content was determined by densitometric measurements of immunoblots of whole cell lysates and normalized to β-actin content. **A**) CGS21680 increased collagen-1 protein (21% ± 5% increase relative to control, $P < 0.05$, n = 7) compared with 100% baseline collagen-1 production. This increase was enhanced in the presence of TNFα (37% ± 18% increase relative to control, $P < 0.05$, n = 5) and inhibited in the presence of IFNγ (8% ± 4% decrease relative to control, P = n.s., n = 7). P-values are derived from 2-way ANOVA with Bonferroni posttest performed as post-hoc analysis. All instances of statistical significance ($P < 0.05$) are displayed in the figure. **B**) TGF-β1 increased collagen-1 production (44% ± 3% increase relative to control, $P < 0.05$, n = 3) by itself and in the presence of TNFα (45% ± 21% increase relative to control, $P < 0.05$, n = 5) and IFNγ (52% ± 22% increase relative to control, $P < 0.05$, n = 3). P-values are derived from 2-way ANOVA with Bonferroni posttest performed as post-hoc analysis. All instances of statistical significance ($P < 0.05$) are displayed in the figure.
Abbreviations: IFNγ, interferon-gamma; TNFα, tumor necrosis factor alpha; A2AR, adenosine A2A receptor; TGF-β1, tumor growth factor beta 1.

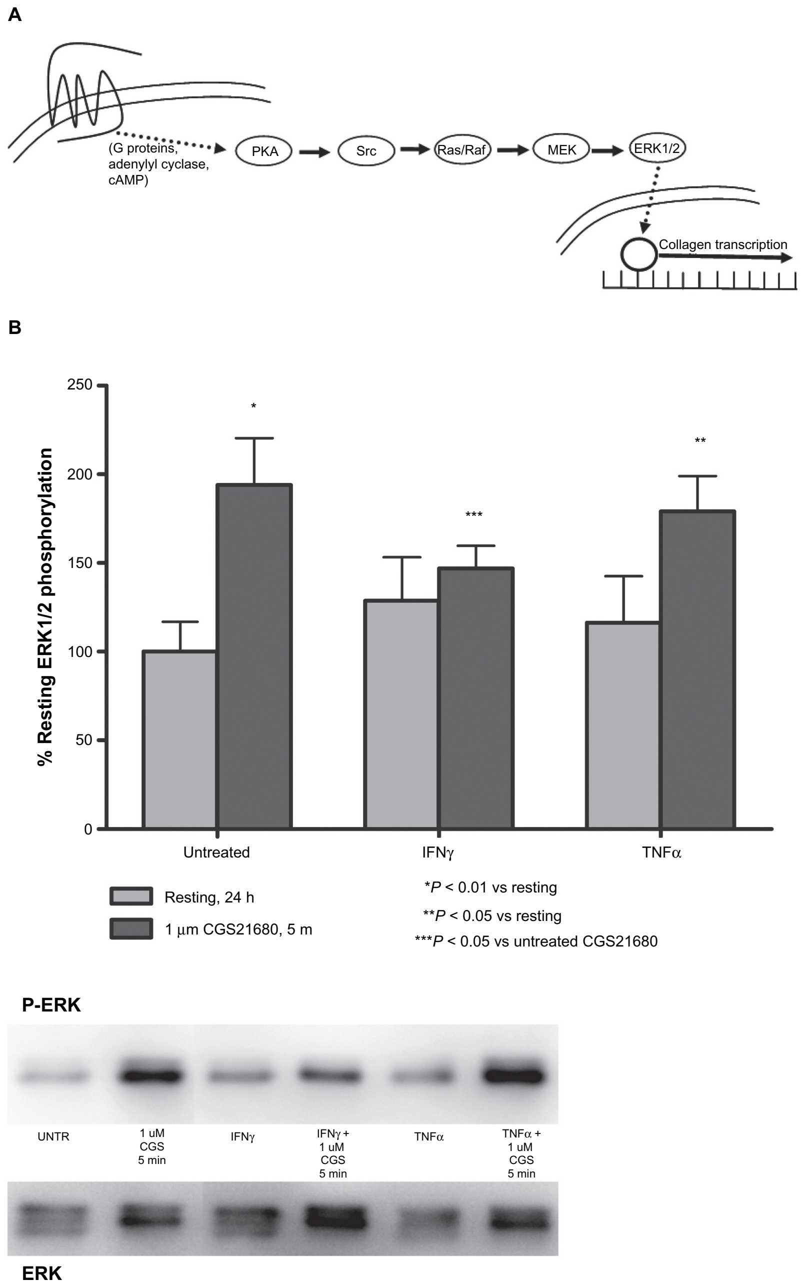

Figure 2 IFNγ impairs the ability of an A2A receptor specific agonist to induce ERK phosphorylation in LX-2 cells. **A**) A schematic representation of the A2A receptor signaling pathway in LX-2 cells. **B**) LX-2 cells were cultured overnight in the presence or absence of IFNγ and TNFα and treated with the A2AR agonist CGS21680 for 5 minutes as described in the Materials and methods section. Phospho-ERK content was determined by densitometric measurements of immunoblots of whole cell lysates and normalized to total ERK content. CGS21680 increased ERK1/2 phosphorylation (94% ± 26% increase relative to control, $P < 0.01$, n = 6) by itself and in the presence of TNFα (70% ± 36% increase relative to control, $P < 0.05$, n = 3) compared with baseline ERK1/2 phosphorylation. This increase was inhibited in the presence of IFNγ (30% ± 20% increase relative to control, P = n.s. vs control and $P < 0.05$ vs untreated CGS21680, n = 6). P-values are derived from 2-way ANOVA with Bonferroni posttest performed as post-hoc analysis. All instances of statistical significance ($P < 0.05$) are displayed in the figure.

Abbreviations: IFNγ, interferon-gamma; TNFα, tumor necrosis factor alpha; cAMP, cyclic AMP; PKA, protein kinase A; ERK1/2, extracellular signal-related protein kinases 1 and 2; A2AR, adenosine A2A receptor.

coupled receptor signaling leads to protein kinase A (PKA) activation and phosphorylation of extracellular signal-related protein kinases 1 and 2 (ERK1/2), which is required for A2A receptor mediated collagen-1 production. To determine if A2A receptor signal transduction is inhibited by IFNγ, we measured the ability of the A2A receptor specific agonist CGS21680 to induce ERK1/2 phosphorylation in the presence and absence of cytokines. As expected, CGS21680 increased ERK1/2 phosphorylation (94% ± 26% increase relative to control, $P < 0.01$, n = 6). This 2-fold increase was transient and peaked 5 minutes after CGS21680 addition. CGS21680-stimulated increases on ERK1/2 phosphorylation were unaffected by the presence of TNFα (70% ± 36% increase relative to control, $P < 0.05$, n = 3). In contrast, IFNγ impaired the ability of CGS21680 to increase ERK1/2 phosphorylation (30% ± 20% increase relative to control, P = n.s. vs 100% control and $P < 0.05$ vs CGS21680 no cytokine, n = 6) (Figure 2B). These results indicate that IFNγ inhibits A2A receptor signal transduction upstream of ERK1/2 phosphorylation IFNγ.

IFNγ impairs the ability of CGS21680 to induce cAMP formation in hepatic stellate cells

cAMP is required for activation of PKA and is located upstream of ERK1/2 in the A2A receptor signal transduction pathway. We have previously demonstrated that IFNγ inhibits A2A receptor-mediated cAMP production in THP-1 cells. In LX-2 cells, CGS21680 increased intracellular cAMP levels in a time-dependent manner, with a peak at 5 minutes after stimulation (48% ± 20% increase relative to control, n = 15). Increased cAMP levels were sustained for at least 20 minutes. IFNγ inhibited A2A receptor-stimulated cAMP production increase (5% ± 21% increase relative to control, $P < 0.05$ vs CGS21680 alone, n = 11) (Figure 3).

IFNγ regulates the expression of adenylyl cyclase message in an isoform specific manner

Kolachala et al have demonstrated that IFNγ inhibits adenosine A2B receptor function in human intestinal epithelial cells by downregulating expression of specific isoforms of adenylyl cyclase.[20] We used realtime RT-PCR to examine expression of all 9 isoforms of adenylyl cyclase in LX-2 cells. mRNA encoding adenylyl cyclase isoform 1 (AC1), normally associated with neuronal cells, was not expressed in LX-2 cells, and message for isoforms AC2, AC4, and AC8 was expressed at very low levels. The message for the remaining 5 adenylyl cyclase isoforms was expressed at much higher levels (data not shown). We measured the effect of IFNγ on adenylyl cyclase mRNA levels for the 5 highly expressed isoforms. mRNA levels for AC6 and AC7 did not change upon IFNγ treatment. mRNA levels for AC5 exhibited a transient increase over time upon IFNγ treatment. mRNA levels for AC3 (0.73 ± 0.13) and AC9 (0.80 ± 0.90) were significantly decreased ($P < 0.05$, n = 6) 24 hours after IFNγ treatment (Figure 4). We conclude that IFNγ downregulates expression of AC3 and AC9.

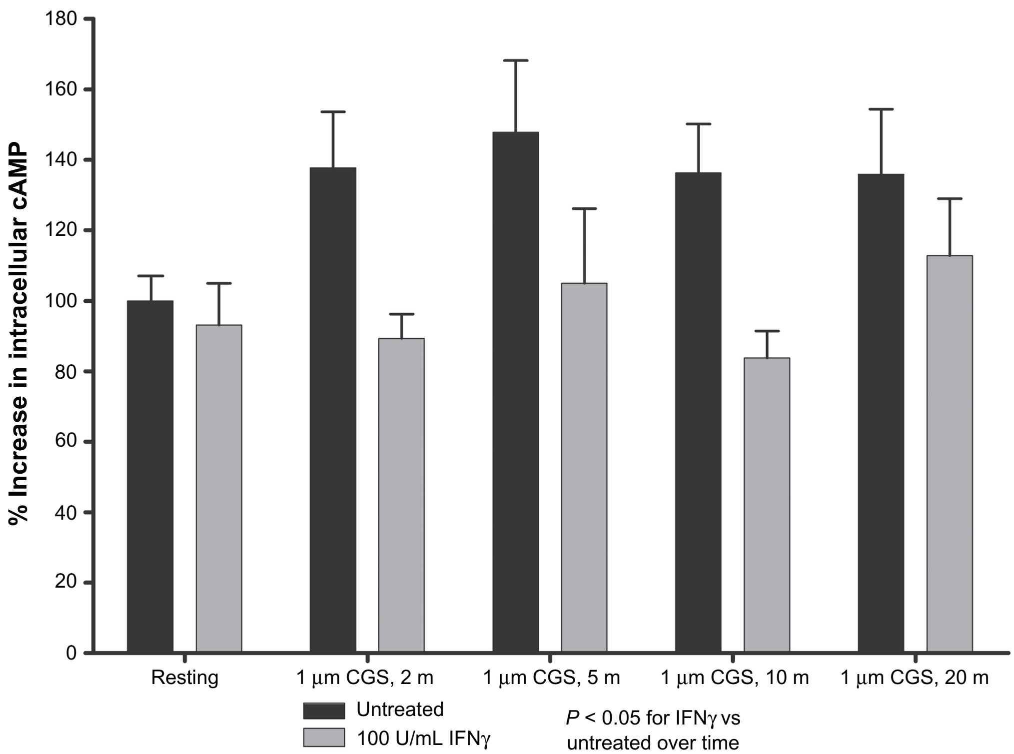

Figure 3 IFNγ impairs the ability of an A2A receptor specific agonist to induce cAMP production in LX-2 cells. LX-2 cells were cultured overnight in the presence or absence of IFNγ and TNFα and treated with the A2AR agonist CGS21680 as described in the Materials and methods section. Intracellular cAMP content was determined by ELISA and normalized to cytosolic protein levels. Basal cAMP levels were 34 pmol/mg intracellular protein. 5 minutes treatment with CGS21680 increased intracellular cAMP levels (48% ± 20% increase relative to control, n = 15) and IFNγ inhibited this increase (5% ± 21% increase relative to control, $P < 0.05$, n = 11). P-values are derived from 2-way ANOVA comparing treatments over time. Post-hoc analysis using Bonferroni posttest does not identify statistical significance between any 2 individual points.
Abbreviations: IFNγ, interferon-gamma; A2AR, adenosine A2A receptor; TNFα, tumor necrosis factor alpha; cAMP, cyclic AMP.

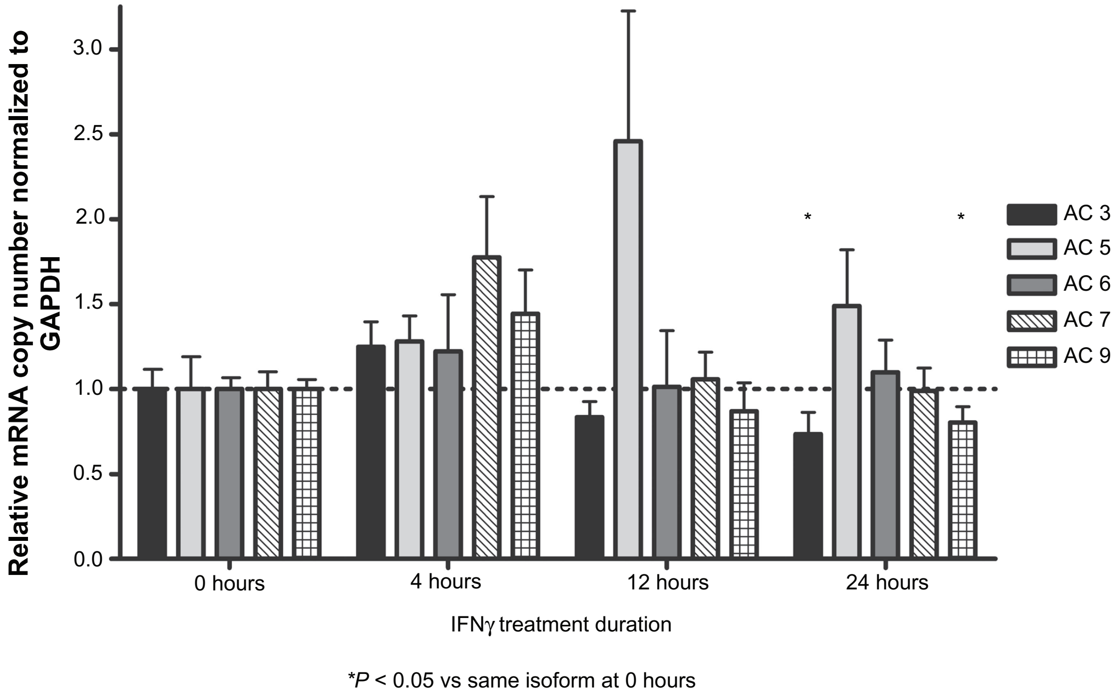

Figure 4 IFNγ downregulates expression of adenylyl cyclase isoforms 3 and 9 in LX-2 cells. Adenylyl cyclase mRNA levels were determined by realtime RT-PCR of whole cell RNA preparations and normalized to GAPDH mRNA levels. LX-2 cells were treated with IFNγ (100 UmL^{-1}), and adenylyl cyclase mRNA levels were determined using C(t) values. 24 hours after IFNγ treatment, mRNA levels for isoforms 3 (0.73 ± 0.13) and 9 (80 ± 0.09) were significantly decreased ($P < 0.05$, n = 6) relative to basal levels. mRNA copy numbers were approximately 1 adenylyl cyclase per 1000 GAPDH. *P*-values are derived from *t*-tests comparing timepoints for a given treatment.
Abbreviations: IFNγ, interferon-gamma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

IFNγ has no effect on the ability of forskolin, a small molecule activator of adenylyl cyclase, to induce cAMP production in LX-2 cells (data not shown). This suggests that AC9 rather than AC3 is required for A2A receptor signaling and function. AC9 is uniquely insensitive to forskolin activation.[22] Our observation that forsoklin is unable to increase collagen-1 production in LX-2 cells supports a requirement for AC9 (data not shown). The inability of forskolin to increase collagen expression in fibroblasts is well established.[32,33]

Downregulation of adenylyl cyclase expression impairs A2A receptor signaling and function

We hypothesized that AC3 or AC9 is required for A2A receptor function. We used siRNA to reduce expression of these 2 isoforms as seen in the Materials and methods section. siRNA knockdown of AC3 decreased AC3 expression (0.40 ± 0.06, n = 3) but not AC9 expression (1.72 ± 0.13, n = 2). siRNA knockdown of AC9 decreased AC9 expression (0.59 ± 0.02, n = 3) but not AC3 expression (1.44 ± 0.17, n = 2) (Figure 5A). We then examined A2A receptor function in these cells. We determined the ability of siRNA to impair CGS21680 induced adenylyl cyclase activity by measuring cAMP concentration. As seen previously, the A2A receptor specific agonist CGS21680 increased intracellular cAMP levels in a time-dependent manner, with a peak at 5 minutes after stimulation (116% ± 46% increase relative to control, n = 4). The knockdown of either AC3 (13% ± 19% increase relative to control, $P < 0.05$ vs CGS21680 alone, n = 4) or AC9 (20% ± 19% increase relative to control, $P < 0.05$ vs CGS21680 alone, n = 4) blocked CGS21680 induced intracellular cAMP production (Figure 5B). Similarly, knockdown of either AC3 (6% ± 3% decrease, n = 5) or AC9 (7% ± 1% decrease, n = 5) abrogated CGS21680-mediated collagen-1 protein production in a statistically significant manner ($P < 0.05$) (Figure 5C). Thus, the knockdown of either AC3 or AC9 prevents signaling at A2ARs necessary for increased collagen production.

IFNγ regulates A2A receptor function in a STAT1 dependent manner

Canonical IFNγ signal transduction is mediated by signal transducers and activators of transcription (STATs), a family of 7 transcription factors.[23,24] To determine whether STATs mediate IFNγ regulation of A2A receptor function, we used siRNA to knock down STAT1 and STAT3 as seen in the Materials and methods section (Figure 6A). Both STAT1 and

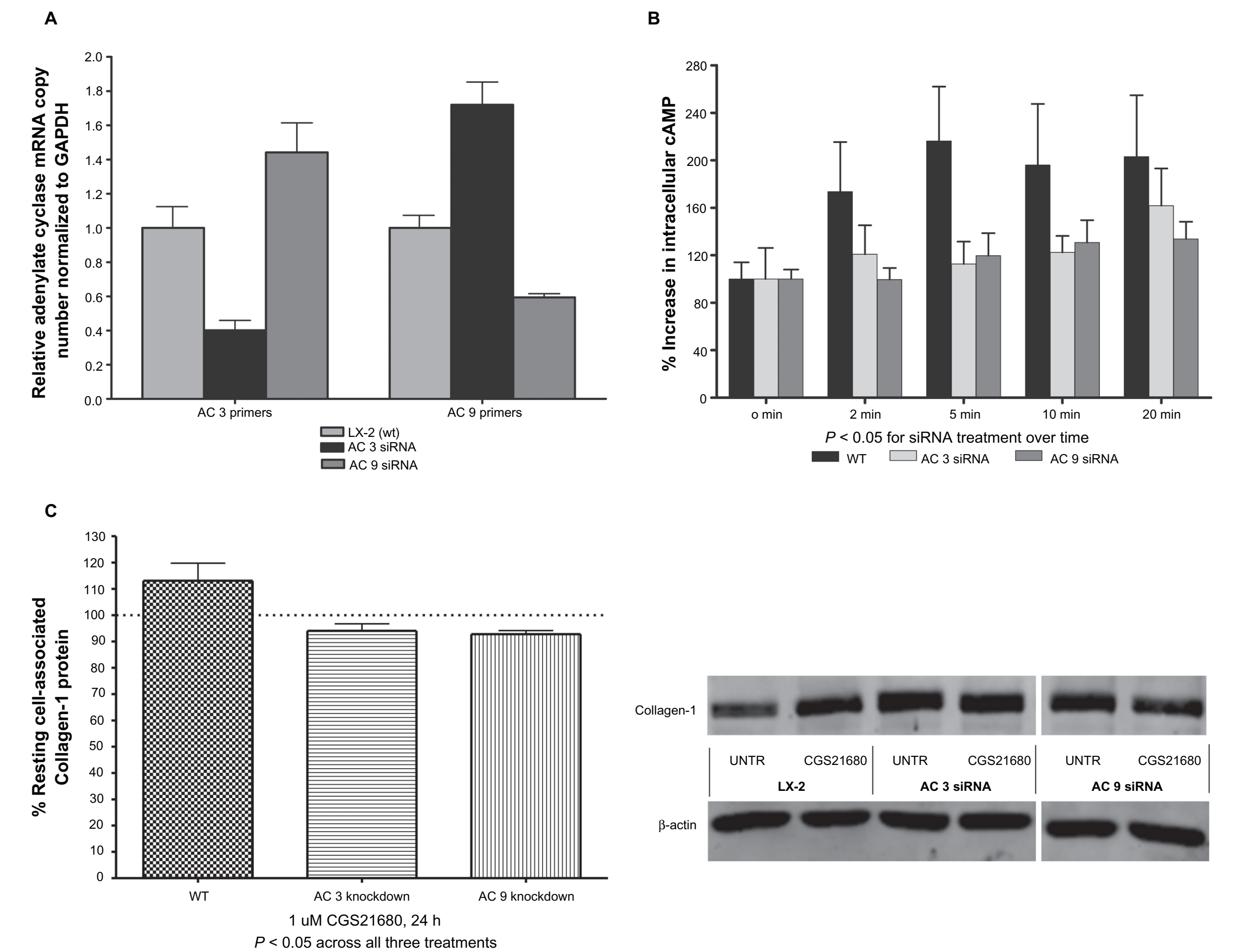

Figure 5 Downregulation of either adenylyl cyclase isoform 3, 6, or 9 inhibits A2A receptor signaling and function in LX-2 cells. Lentiviruses containing siRNA constructs were used to create three separate cell lines, each with impaired expression of a different adenylyl cyclase isoform. **A**) Cellular adenylyl cyclase mRNA content was determined by realtime QPCR and normalized to GAPDH levels. siRNA knockdown of AC3 decreased AC3 expression (0.40 ± 0.06, n = 3) but not AC9 expression (1.72 ± 0.13, n = 2) relative to basal levels. siRNA knockdown of AC9 decreased AC9 expression (0.59 ± 0.02, n = 3) but not AC3 expression (1.44 ± 0.17, n = 2) relative to basal levels. mRNA copy numbers were approximately 1 adenylyl cyclase per 1000 GAPDH. **B**) A2ARs were activated by incubation with the specific agonist CGS21680 (1 μM). Intracellular cAMP content was determined by ELISA and normalized to cytosolic protein levels. Basal cAMP levels were 42 pmol/mg intracellular protein. The 5 minute treatment with CGS21680 increased intracellular cAMP (116% ± 46% increase relative to control, n = 4). Knockdown of AC3 (13% ± 19% increase relative to control, $P < 0.05$, n = 4) or AC9 (20% ± 19% increase relative to control, $P < 0.05$, n = 4) inhibited CGS21680 increased intracellular cAMP. *P*-values are derived from 2-way ANOVA for treatment groups over time. Post-hoc analysis using Bonferroni posttest does not identify statistical significance between any 2 individual points. **C**) A2ARs were activated by incubation with the specific agonist CGS21680 (1 μM) for 24 hours. CGS21680 increased collagen-1 protein expression (13% ± 7%, n = 5). Knockdown of AC3 (–6% ± 3%, n = 5) or AC9 (–7% ± 1%, n = 5) inhibited CGS21680 induced collagen-1 protein expression in a statistically significant manner ($P < 0.05$). *P*-values are derived from 2-way ANOVA with Bonferroni posttest performed as post-hoc analysis. All instances of statistical significance ($P < 0.05$) are displayed in the figure.
Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; A2AR, adenosine A2A receptor; cAMP, cyclic AMP.

STAT3 are phosphorylated by IFNγ in LX-2 cells, while the other isoforms are unaffected (data not shown). We previously demonstrated that ERK1/2 phosphorylation is required for A2A receptor mediated collagen-1 production.[18] We measured A2A receptor mediated ERK1/2 phosphorylation in STAT knockdown cells in the presence and absence of IFNγ.

CGS21680 increased ERK phosphorylation (32% ± 8% increase relative to control, $P < 0.05$, n = 3) and the presence of IFNγ again prevented A2A receptor mediated ERK phosphorylation (13% ± 5% increase relative to control, P = n.s., n = 3). In LX-2 cells with impaired STAT3 expression, CGS21680 increased ERK phosphorylation to levels similar to wild type cells (44% ± 19% increase relative to control, $P < 0.01$, n = 3) and the presence of IFNγ prevented A2A receptor mediated ERK phosphorylation (19% ± 18% increase relative to control, $P > 0.05$, n = 3). In LX-2 cells with impaired STAT1 expression, CGS21680 increased ERK phosphorylation similar to wild type cells (46% ± 20% increase relative to control, $P < 0.01$, n = 3), but STAT1 knockdown abrogated IFNγ mediated inhibition of A2A receptor mediated ERK phosphorylation (44% ± 20% increase relative to control, $P < 0.01$, n = 3)

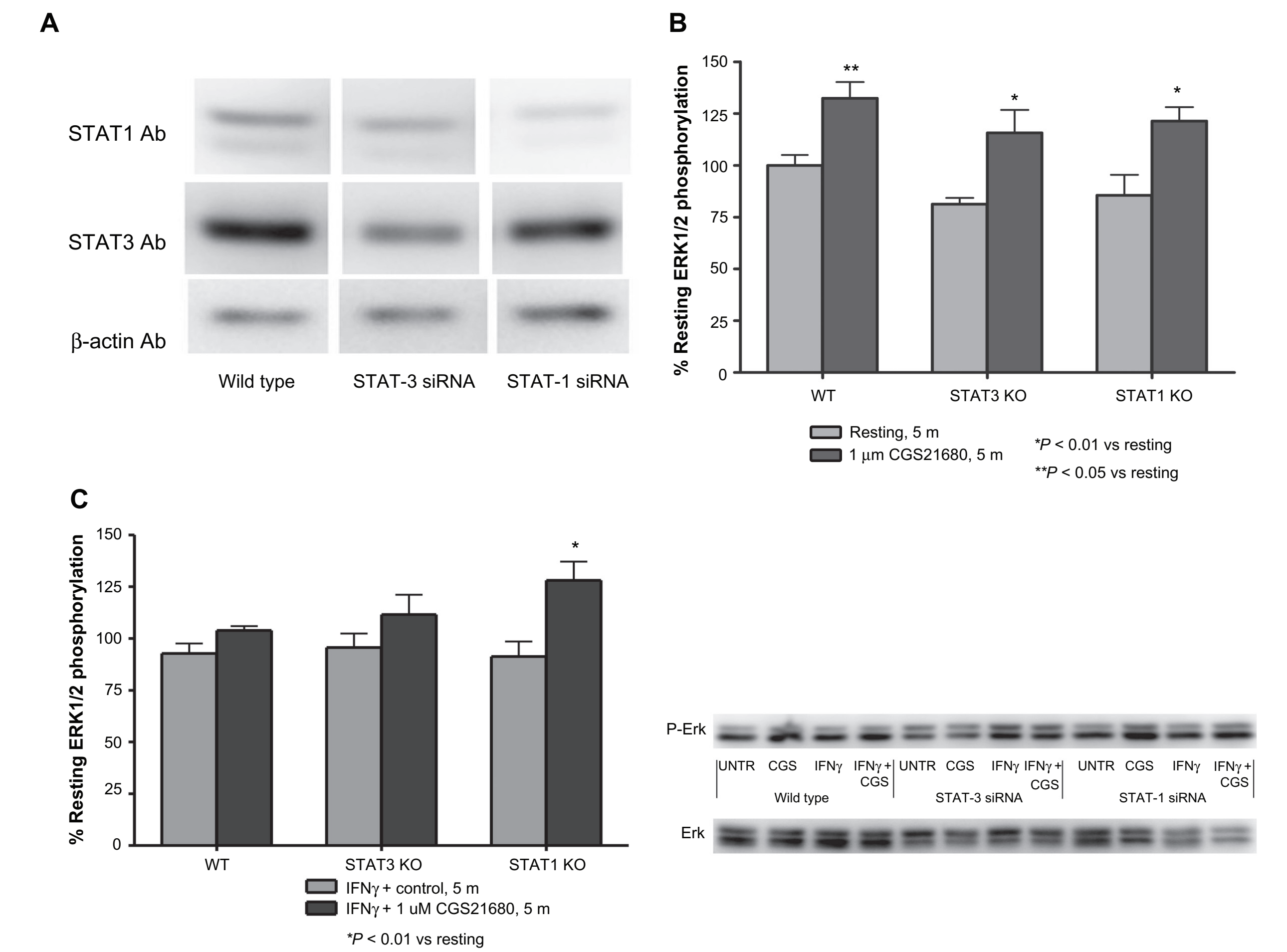

Figure 6 IFNγ has no effect on the ability of CGS21680 to induce ERK phosphorylation in LX-2 cells with impaired STAT1 expression. **A**) STAT1 and STAT3 content was determined by immunoblots of whole cell lysates normalized to β-actin content. siRNA knockdown of STAT3 decreased STAT3 expression but not STAT1 expression. siRNA knockdown of STAT1 decreased STAT1 expression but not STAT3 expression. All pictures are from the same gel, which was stripped and reprobed. **B**, **C**) LX-2 cells were cultured overnight in the presence or absence of IFNγ and TNFα and treated with the A2AR agonist CGS21680 for 5 minutes as described in the Materials and methods section. Phospho-ERK content was determined by densitometric measurements of immunoblots of whole cell lysates and normalized to total ERK content. CGS21680 increased ERK phosphorylation in wild-type cells (32% ± 8% increase relative to control, $P < 0.05$, n = 3) was unaffected by knockdown of STAT3 (44% ± 19% increase relative to control, $P < 0.01$, n = 3) and STAT1 (46% ± 20% increase relative to control, $P < 0.01$, n = 3). IFNγ inhibited CGS21680 increased ERK phosphorylation in wild-type cells (13% ± 5% increase relative to control, P = n.s., n = 3) and STAT3 knockdown cells (19% ± 18% increase relative to control, $P < 0.05$, n = 3). Knockdown of STAT1 abrogated inhibition (44% ± 20% increase relative to control, $P < 0.01$, n = 3). P-values are derived from 2-way ANOVA with Bonferroni posttest performed as post-hoc analysis.

Abbreviations: IFNγ, interferon-gamma; STAT1, signal transducer and activator of transcription 1; TNFα, tumor necrosis factor alpha; ERK1/2, extracellular signal-related protein kinases 1 and 2; A2AR, adenosine A2A receptor; STAT3, signal transducer and activator of transcription 3.

(Figures 6B and 6C). We conclude that STAT1 expression is necessary for IFNγ mediated regulation of A2A receptor function. The inability of either siRNA to affect A2A receptor signal transduction controls for non-specific siRNA effects on A2A receptor signaling and function.

Discussion

We report here that IFNγ inhibits the ability of the A2A receptor specific agonist CGS21680 to induce collagen production, ERK phosphorylation, and cAMP production in LX-2 cells. IFNγ impairs adenylyl cyclase expression, and adenylyl cyclase transduces the A2A receptor signal for increased collagen production. Moreover, we have found that the JAK/STAT pathway mediates the effect of IFNγ on A2A receptor function. We conclude that IFNγ inhibits the function of the A2AR by downregulating the expression of AC3 and AC9 in a STAT1 mediated fashion (Figure 7). While both IFNγ and TNFα decrease basal collagen-1 protein levels in LX-2 cells, they have opposite effects on hepatic fibrosis in vivo. Increased TNFα levels are associated with increased hepatic fibrosis in human and animal models.[21] This suggests that the clinically relevant effect of IFNγ on hepatic fibrosis is one it does not share with TNFα, such as an ability to inhibit A2A receptor function. In vivo protection from hepatic fibrosis afforded by IFNγ may therefore be due, at least in part, to inhibition of A2A receptor mediated collagen production.

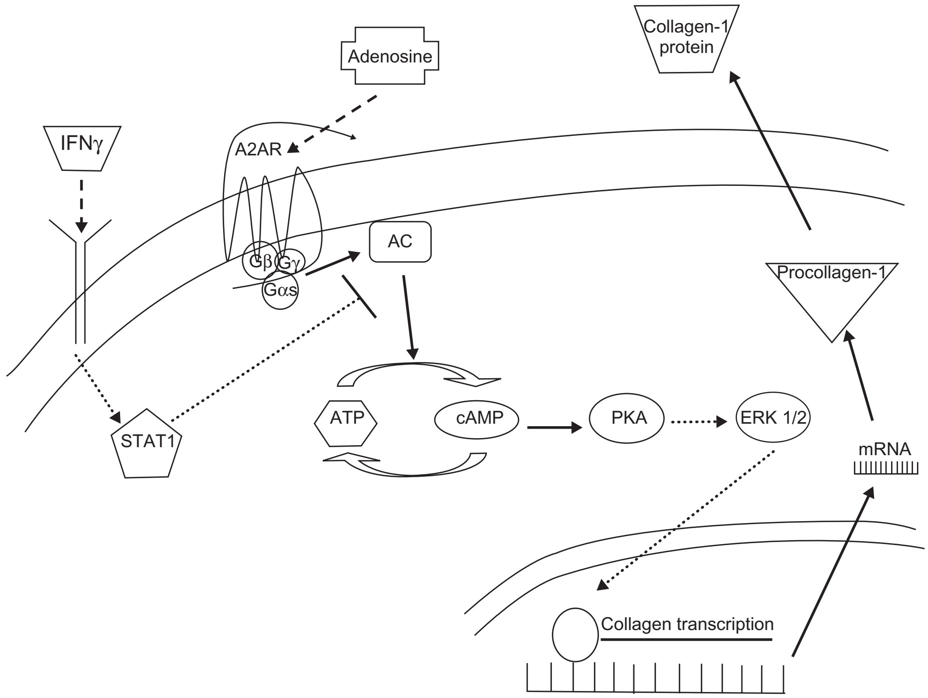

Figure 7 Proposed model for the mechanism by which IFNγ impairs A2A receptor signaling and function.
Abbreviations: IFNγ, interferon-gamma; A2AR, adenosine A2A receptor; STAT1, signal transducer and activator of transcription 1; cAMP, cyclic AMP; PKA, protein kinase A; ERK1/2, extracellular signal-related protein kinases 1 and 2.

Our data suggest a role for multiple adenylyl cyclase isoforms in A2A receptor signaling. Cooper and Crossthwaite summarize recent evidence demonstrating that oligomerization is a key feature in adenylyl cyclase function. Increases in cAMP levels are tightly localized to membrane microdomains whose organization is determined by the ability of molecules including adenylyl cyclase to form higher-order structures.[25] Oligomerization is necessary to recruit adenylyl cyclase to multimeric signaling assemblies required for efficient GPCR signal transduction.[26] We speculate that AC3 and AC9 oligomerization is required for A2A receptor signal transduction. Both isoforms may be necessary to localize adenylyl cyclase to the A2A signaling module. AC3 and A2A are observed in membrane microdomains, while AC9 is not. Alternatively, oligomerization may increase adenylyl cyclase activity to levels needed to achieve sufficiently high levels of cAMP for downstream signaling. Adenylyl cyclase oligomerization in the presence of Gαs was demonstrated to enhance enzymatic function.[27] To the best of our knowledge, this is the first suggestion that these two particular adenylyl cyclase isoforms may oligomerize in vivo.

Further study examining the localization of the molecules involved in A2A receptor signal transduction is warranted. We are particularly interested in the co-localization of AC3 and AC9, as well as changes in localization of both isoforms upon AC3 and AC9 knockdown. Knockdown of adenylyl cyclase may also disrupt the function of other A2A receptor signaling mediators. Expression of different GPCR signaling molecules are all linked. For example, siRNA mediated knockdown of Gβ protein alters Gα and adenylyl cyclase protein expression.[22] Adenylyl cyclase isoforms also cross-regulate each other. For example, AC6 can regulate Ca^{2+} influx and Ca^{2+} influx AC1, AC3, and AC8 while inhibiting AC5 and AC6.[25,28]

It remains possible that IFNγ regulates adenylyl cyclase activity in addition to expression. Adenylyl cyclase activity is regulated post-transcriptionally by molecules such as regulator of G protein signaling (RGS), PKA, PKC, and calmodulin kinase, which may be affected by IFNγ treatment.[22] IFNγ could also increase the activity or expression of phosphodiesterases (PDEs). PDEs regulate adenylyl cyclase function by catalyzing the hydrolysis of cAMP into AMP.[29,30] Increased PDE activity would inhibit A2A receptor signaling by preventing cAMP from activation PKA and Epac. PDEs are unlikely to mediate the effect of IFNγ on A2A receptor function, however, since IFNγ was shown to downregulate expression of multiple PDEs, as well as RGS2, in pancreatic stellate cells.[31] Decreased expression of regulatory molecules such as PDEs would increase A2A function rather than inhibit it.

Previous studies in our lab have demonstrated the ability of IFNγ to regulate A2A receptor function in THP-1 human monocyte cell line.[13] It remains to be determined whether IFNγ also downregulates adenylyl cyclase expression in these cells. It is interesting that while IFNγ has an opposite effect from TNFα on A2A receptor function in THP-1 cells, it has a completely different mechanism of action. Previous research in our laboratory has demonstrated that TNFα enhances A2A receptor activity by impairing GRK2 and β-arrestin mediated receptor desensitization in the THP-1 human monocyte cell line.[14] Treatment with CGS21680 increased membrane bound GRK2 in LX-2 cells, but the presence of IFNγ had no effect on CGS21680 induced GRK2 translocation (data not shown). Consistent with observations in THP-1 cells, TNFα blocked A2A receptor-mediated GRK2 translocation to the cell membrane (data not shown). Unlike TNFα, IFNγ has no effect on GRK2 translocation and A2A receptor desensitization. While TNFα impairs A2A receptor desensitization, IFNγ has no effect on desensitization, but directly impairs the A2A receptor signal transduction cascade.

We have shown that the effect of IFNγ on A2A receptor function is mediated by STAT1, but it remains unclear whether or not STAT1 directly suppresses adenylyl cyclase expression. Sequence analysis of adenylyl cyclase promoter regions failed to identify a consensus binding site for STAT1, supporting the hypothesis that STAT1 regulates another transcription factor with the ability to regulate adenylyl cyclase expression. Further analysis is necessary to determine putative regulatory factor binding sites in the adenylyl cyclase promoter region in order to identify this unknown intermediary.

Studies in patients with hepatitis B[32] and rats with CCl4- induced hepatic fibrosis[33,34] have shown that IFNγ exerts a protective effect on liver fibrosis in vivo, but have not considered a potential role for the adenosine receptor.[32,35] IFNγ prevents activation of hepatic stellate cells both in vitro[36] and in vivo.[37] However, our results demonstrate a novel role for IFNγ in regulating hepatic stellate cell activity post- activation. This may prove clinically relevant, as the components of the adenosine receptor signaling pathway provide new targets for drug development to treat hepatic fibrosis.[38] Furthermore, a role for adenosine receptors in fibrosis is not limited to the liver. Our lab has demonstrated a role for the A2A receptor in skin fibrosis[39] and other adenosine receptors are relevant to the progression of cardiac[40] and pulmonary fibrosis.[7] It is important to explore the ability of IFNγ to regulate adenosine receptor function in these other tissues. Activation of the IFNγ receptor or its signaling pathway could be used to treat fibrosis in these tissues as well.

We have identified a novel mechanism for regulation of adenosine receptor function by IFNγ in the context of hepatic fibrosis. Ultimately, our results identify adenylyl cyclase activity and expression as a key point of regulation for adenosine receptor function and a potentially valuable therapeutic target for disease models involving adenosine receptor signaling, such as hepatic fibrosis.

Acknowledgments

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Conflicts of interest

Eric T Block, PhD

None.

Bruce N Cronstein, MD

Intellectual property

- Patents on use of A2AR agonists to promote wound healing and use of A2A receptor antagonists to inhibit fibrosis.
- Patent on use of adenosine A1 receptor antagonists to treat osteoporosis and other diseases of bone.
- Patent on the use of adenosine A1 and A26 receptor antagonists to treat fatty liver.
- Patent on the use of A2AR agonists to prevent prosthesis loosening.

Consultant (within the past two years), all <\$10,000

- Cypress Bioscience, Inc.
- King Pharmaceutical (licensee of patents above)
- CanFite Biopharmaceuticals
- Bristol-Myers Squibb
- Celizome
- Tap Pharmaceuticals
- Prometheus Laboratories
- Regeneron (Westat, DSMB)
- Sepracor
- Amgen
- Endocyte
- Protalex
- Allos, Inc.
- Combinatorx
- Kyowa Hakka

- Hoffman-LaRoche
- Savient
- Avidimer Therapeutics

Stock

CanFite Biopharmaceuticals received for membership in Scientific Advisory Board.

Grants

- King Pharmaceuticals
- NIH
- Vilcek Foundation
- Board Member: Vilcek Foundation

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