The Adenosine A2A Receptor Agonist UK-432,097 Stimulates Expression of Anti-Atherogenic Reverse Cholesterol Transport Proteins

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Abstract

Methotrexate, a disease modifying anti-rheumatic drug, has been shown to reduce cardiovascular events in rheumatoid arthritis patients. Our group discovered that one of the ways methotrexate is athero-protective is through an increase of adenosine occupying the adenosine A2A receptor (A2A R), which increases reverse cholesterol transport (RCT) mechanisms such as the expression of ATP-binding cassette transporter (ABC) A1 and G1, as well as cholesterol 27-hydroxylase – effectively impeding lipid overload. Methotrexate is associated with safety issues, such as liver and/or renal dysfunction, that can limit its use. Therefore, we sought to examine the efficacy of UK-432, 097, a specific A2A R agonist developed as an inhaled drug and therefore known to possess an established safety profile in humans. We hypothesized that UK-432,097 would increase RCT proteins and thereby prevent excess cholesterol accumulation in macrophages. This study shows that UK-432, 097 increases the expression of 4 RCT proteins: ABCA1, ABCG1, liver x receptor (LXR) and 27-hydroxylase and that this effect is mediated through the A2A R. Findings in this report support the concept that A2A R ligation improves cellular cholesterol metabolism and provides evidence for exploring it as a therapeutic option for reducing atherosclerosis.

Keywords — adenosine agonist, atherosclerosis, cardiovascular disease, UK-432,097.

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I. INTRODUCTION

Cholesterol accumulation within atherosclerotic plaque occurs when cholesterol efflux from the arterial wall fails to keep up with influx.1 Cholesterol efflux occurs through the reverse cholesterol transport (RCT) pathway by which cholesterol within peripheral cells is returned to the liver for catabolism and excretion.1 Impaired RCT leads macrophages to transform into foam cells, a cell type overloaded with cholesterol that is a key element of atherosclerotic lesions.3,4

Major participants in macrophage RCT include the ATP-binding cassette transporters (ABC) A1 and ABCG1, the liver X receptor (LXR) and the cytochrome P450 cholesterol 27-hydroxylase. ABCA1 is a major gatekeeper of RCT.5,6 ABCA1 is a trans-membrane protein responsible for the efflux of cholesterol and phospholipids to apolipoproteins such as apoA1 and apoE.7 ApoA1 is used in the production of high-density lipoprotein (HDL). HDL, in turn, carries cholesterol from body tissues to the liver where it is removed from the blood. The removal of excess cholesterol from cells by the ABCA1 transport protein is critical for balanced cholesterol levels and maintenance of cardiovascular health.8 ABCG1 is responsible for mediating cholesterol efflux into mature HDL in macrophages.9 ABCA1 and ABCG1 can work together to accomplish RCT.10

ABCA1 and ABCG1 are LXR target genes.11 Oxyesters, oxygenated derivatives of cholesterol, are endogenous ligands for LXR. The oxyester 27-hydroxycholesterol is produced by the 27-hydroxylase enzyme and, through LXR activation, upregulates ABCA1 and ABCG1.12,13 27-Hydroxycholesterol activates cholesterol-utilizing enzymes via LXR to induce genes acting on cholesterol efflux, transport, and excretion. In addition to its action on LXR, 27-hydroxycholesterol has a number of other athero-protective properties. It exits cells through lipid membranes orders of magnitude faster than cholesterol and it behaves like a statin drug by inhibiting the rate-limiting enzyme in cholesterol synthesis, hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase.14

Adenosine, a purine nucleoside and critical modulator of inflammation, has been shown previously to have atheroprotective properties mediated through activation of the
Adenosine A₂₃ receptor (A₂₃R). A₂₃R ligation reduces the development of foam cells and augments the expression of the ABCA1 and 27-hydroxylase proteins. Methotrexate, a disease modifying anti-rheumatic drug used to treat rheumatoid arthritis, acts on cells to promote adenosine release. It has long been known that methotrexate reduces cardiovascular events in rheumatoid arthritis patients and this may be due to adenosine effects not only on inflammation, but on cholesterol metabolism as well. Previous work by our group and others has given support to the concept that regulation of RCT protein expression by A₂₃R agonism could provide a valuable approach in improving macrophage cholesterol handling, thus diminishing atherosclerosis.

UK-432,097, a highly specific A₂₃R agonist, has a high binding affinity for A₂₃R and stabilizes the receptor upon binding. UK-432,097 was originally developed as an inhaled treatment for inflammatory symptoms of chronic obstructive pulmonary disease, but later discontinued due to poor efficacy. This agonist agent, although administered to human subjects, has not been evaluated for its atheroprotective properties. The present study demonstrates augmentation of RCT gene expression and improved cholesterol export in human macrophages treated with UK-432,097.

II. METHODS

Cells and Reagents

THP-1 human monocytes were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media and supplementary reagents were obtained from Invitrogen (Grand Island, NY). Phorbol 12-myristate 13-acetate (PMA) and Oil red O were purchased from Sigma-Aldrich (St. Louis, MO).

The selective A₂₃R agonist 2-p-(2-carboxyethyl)phenethylamino-5-N-ethylcarboxamidooxycytidine (CGS-21680) was purchased from Sigma Chemical Co (St. Louis, MO).

UK-432,097 was purchased from Axon Medchem (Groningen, The Netherlands).

The selective A₂₃R antagonist 4-(2-$\text{-}[7$-aminooxy$-2$-furyl]$-1, 2, 4$-\text{triazolo}[2,3$-a$]$-1, 3, 5$-\text{triazin}$-5$-lamino$-ethyl$)phenol (ZM-241385) was purchased from Tocris Cookson (Bristol, UK).

Trizol reagent was purchased from Invitrogen (Grand Island, NY).

All reagents for reverse transcription-polymerase chain reaction (RT-PCR) were purchased from Applied Biosystems (Chicago, IL). FastStart SYBR Green Master mix for the quantitative real-time polymerase chain reaction (QRT-PCR) was obtained from Roche Applied Science (Indianapolis, IN). Primers used in amplification reactions were generated by Sigma-Genosys (The Woodlands, TX).

The BCA Protein Assay Kit and all reagents for Western blot protein detection were purchased from Thermoscientific Pierce Biotechnology Inc. (Rockford, IL).

Anti-cholesterol 27-hydroxylase antibody is an affinity-purified rabbit polyclonal anti-peptide antibody raised against residues 15-28 of the 27-hydroxylase protein. Rabbit anti-human ABCA1 (sc-20794) and LXR$α$(sc-20) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human ABCG1 (ab-36969) and β-actin (ab-6276) antibody were purchased from Abcam Inc. (Cambridge, MA). Donkey anti-rabbit horseradish peroxidase (HRP) linked antibody, used as a secondary antibody, was purchased from GE Healthcare Biosciences (Piscataway, NJ).

Cell Culture

The THP-1 human monocyte cell line was grown in the monocyctic form in suspension in RPMI 1640 medium with 10% fetal calf serum (FCS) at 37°C in a 5% CO₂ atmosphere. To facilitate differentiation into macrophages, THP-1 monocytes were treated with 100nM PMA for 48h. When differentiated phenotype was achieved, the PMA-containing medium was removed and replaced with fresh complete RPMI 1640, supplemented with 10% FCS. Studies were performed at a density of $1 \times 10^{6}$ cells/ml.

Experimental Conditions

THP-1 macrophages were resuspended in RPMI 1640 growth medium and incubated in 12-well plates for 3, 6, and 18h under the following conditions: (1) media alone (untreated control); (2) dimethyl sulfoxide (DMSO) vehicle (solvent control); (3) UK-432,097 (100 nM); (4) ZM-241385 (1μM, 1 h) + UK-432,097 (100 nM). For protein expression analysis, cells were incubated for 24h under conditions 1-4 as above and two additional condition: (5) CGS-21680 (1 μM); and (6) ZM-241385 (1 μM, 1 h) + CGS-21680 (1 μM). After incubation, media was aspirated, and cellular RNA and protein were isolated.

RNA Isolation and Gene Expression Analysis by QRT-PCR

Total RNA was isolated by adding 1 ml Trizol reagent per $10^6$ cells. RNA concentration was determined by measuring the absorbance at 260 and 280 nm wavelengths by ultraviolet spectrophotometry (Hitachi U2010).

cDNA was copied from 1 μg of total RNA using Murine Leukemia Virus reverse transcriptase primed with oligo dT in an Eppendorf Mastercycler®-personal PCR thermocycler (Eppendorf, Hamburg, Germany). Equal amounts of cDNA were taken from each RT reaction mixture for PCR amplification using specific primers (Table 1). QRT-PCR analysis was performed using the FastStart SYBR Green Reagent Kit according to the manufacturers' instructions on the Roche Light Cycler 480 (Roche Applied Science, Indianapolis, IN). Each reaction was done in triplicate. The amounts of PCR products were determined using Roche Applied Science software, provided by the manufacturer. Fluorescence emission spectra were monitored and analyzed. PCR products were measured by the threshold cycles ($C_T$), at which specific
fluorescence becomes detectable. The $C_T$ value for each gene was normalized to that for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and the relative expression level was calculated as the mean value of the untreated THP-1 as 1.

Non-template controls were included for each primer pair to check for significant levels of any contaminants. A melting-curve analysis was performed to assess the specificity of the amplified PCR products.

***Protein extraction and Western blot analysis

Total cell lysates were prepared for Western blotting using RIPA lysis buffer (98% PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]), containing protease inhibitor cocktail (Sigma) [10µl per 100µl of RIPA buffer]. Cell pellets from each condition were incubated on ice for 35 min. Supernatants were collected after centrifuging at 10,000g at 4°C for 10 min. The quantity of protein in each supernatant was measured by absorption at 560nm using a Hitachi U2010 spectrophotometer.

Protein samples (20µg/lane) were boiled for 5 minutes, fractionated on 8% SDS-PAGE, and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was stained with Ponceau red (Sigma-Aldrich) to verify uniformity of protein loading in each lane.

The membrane was blocked for 1 hour at room temperature in blocking solution (5% nonfat dry milk (Bio-Rad) in 1X Tris-buffered saline/1% Tween 20 [TTBS]) and then immersed in different dilutions of primary antibody for protein detection. To detect and quantify expression of 27-hydroxylase, a 1:300 dilution of primary antibody in blocking solution was used overnight at 4°C. 1:500 dilutions of primary antibody were used for detection of ABCA1, ABCG1 and LXR-α. The following day, the membrane was washed and then incubated in a 1:5000 dilution of ECL HRP-linked donkey anti-rabbit antibody in blocking solution. As a control, on the same transferred membrane, beta-actin was detected at 1:1000 dilution of antibody. The immunoreactive proteins were detected using Pierce ECL Western Blot substrate system, and film development in SRX-101A (Konica Minolta Holdings, Inc., Tokyo, Japan). Stripping and re-probing of the membranes were performed according to the manufacturer’s protocol (Thermoscientific, Rockford, IL). Band intensities for Western blot protein samples were quantified using Kodak Digital Science 1D, version 2.0.3, after imaging with Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

Analysis of oxidized low density lipoprotein (oxLDL) accumulation in THP-1 macrophages

THP-1 monocytes (10⁶ cells per ml) were transferred into 8-well glass-chamber slides, and treated with PMA (100nM, 48h, 37°C) to stimulate differentiation into macrophages. Differentiated macrophages were cholesterol-loaded with 50 µg/ml oxLDL (Inracel, Frederick, MD) for 24 h, and subjected to conditions described above in the presence of 5 µg/ml 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanin (DiI)-oxLDL (Inracel, Frederick, MD) for 3h. After incubation, accumulation of Dil-oxLDL in cells was determined by fluorescent intensity of accumulated Dil-oxLDL with a Nikon A1 microscope unit with 40X magnification and photographed with a DS-Ri1 digital camera. Fluorescent intensity was quantified from at least 3 random fields (1024x1024 pixels) per slide, from 3 slides per experimental condition and graphed.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism, version 5.01. All data were analyzed by one-way analysis of variance, and pairwise multiple comparisons were made between control and treatment conditions using Bonferroni correction. $P$ values less than 0.05 were considered significant.

III. RESULTS

UK-432,097 increases expression of RCT proteins and A32R blockade negates this effect in THP-1 human macrophages

In all experiments mRNA and protein expression levels in THP-1 macrophages with RPMI medium alone were set at 100%.

Incubation of THP-1 macrophages with the A32R agonist UK-432,097 (100nM) increased expression of mRNA for ABCA1, ABCG1, LXRα, and 27-hydroxylase (Figure 1).

After 6h of exposure to UK-432,097 in cell culture, mRNA and protein levels of ABCA1 increased significantly by 88.75±5.4% and by 56.34±12.4% above control (n=3, P<0.01), respectively (Figure 2). ABCG1 message and protein increased by 58.42±6.32% and 65.45±5.24% vs. control (n=3, P<0.01), respectively (Figure 3).

For THP-1 macrophages, the effect of UK-432,097 on expressions of 27-hydroxylase and LXRα reached significance after 18h of incubation. Thus, at 18h, mRNA and protein expression of 27-hydroxylase were increased by 46.45±3.4% and 50.27±8.9% (n=3, P<0.01), respectively (Figure 4). Message and protein level of LXRα was upregulated to 155.80±4.9% and 157.98±12.9% of control (n=3, P<0.01), respectively (Figure 5).

The same pattern was shown for regulation of proteins involved in cholesterol efflux by CGS-21680 (Figures 2-5), used as the gold standard for A32R activation in numerous studies from our lab and others.

Expression of targeted RCT proteins in UK-432,097/ ZM-241,385 treated macrophages decreased from 3 to 18 hours due to A32R occupancy by the antagonist (Figures 2-5).
Accumulation of oxLDL is reduced by UK-432,097 and A2AR blockade with ZM-241-385 abrogates this effect in THP-1 human macrophages

As additional evidence for the anti-atherogenic properties of UK-432,097, we observed a significant downregulation of oxLDL uptake by THP-1 macrophages treated with pro-atherogenic lipids. Accordingly, THP-1 macrophages incubated in the presence of UK-432,097 internalized significantly less Dil-oxLDL compared to control cells (Figure 6). Thus, exposure of macrophages to UK-432,097 decreased oxLDL uptake by 28.9% (P<0.01, n=5). Pre-incubation of the THP-1 macrophages with ZM-241,385 prevented changes in oxLDL uptake so that oxLDL internalization was not affected by UK-432,097 under conditions where the A2AR was blocked (Figure 6).

TABLE I
THE LIST OF SPECIFIC PRIMERS USED FOR QRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>F 5’-GAACTGATCATGACATGGGC-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-GATCAAGCCATGGCTGTAG-3’</td>
</tr>
<tr>
<td>ABCG1</td>
<td>F 5’-CAGGAAGATGAGACTGTGG-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-GAAATGGGGATGAGAAG-3’</td>
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<tr>
<td>27-hydroxylase</td>
<td>F 5’-AAGCGATACCTGGATGGTG-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-TGTTGGATGTGGTTCCACT-3’</td>
</tr>
<tr>
<td>LXRα</td>
<td>F 5’-GGGCCAGCCCCAAATGCTG-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-GCATCCGTGGGAAATCAGTGGCG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F 5’-ACCATCAGCCCTGCTCTAC-3’</td>
</tr>
<tr>
<td></td>
<td>R 5’-CCTGTTGCTGTAGCCAAAT-3’</td>
</tr>
</tbody>
</table>

Fig. 1. Time course analysis of UK432,097 effect on RCT protein message level in cultured THP-1 human macrophages. Cells were incubated in the presence of UK-432,097 for 3h, 6h and 18h. After incubation, media was aspirated, total RNA was isolated and reverse transcribed into cDNA. Gene expression analysis was performed using QRT-PCR, normalized to the expression of the housekeeping gene (GAPDH) and graphed.

Fig. 2. Western blot illustrating effect of UK432,097 on protein expression in THP-1 human macrophages. Western blot analysis using specific anti-human antibodies to ABCA1, ABCG1, LXRα and 27-hydroxylase after 24h incubation of THP-1 macrophages under the following conditions: (1) dimethyl sulfoxide (DMSO) vehicle (solvent control); (2) UK-432,097 (100 nM); (3) ZM-241385 (1μM) + UK-432,097 (100 nM); (4) CGS-21680 (1 μM); (5) ZM-241385 (1 μM, 1 h) + CGS-21680 (1 μM). β-actin was detected on the same membrane for normalization.
Figure 3. Effect of UK432,097 on the mRNA level (A) and protein expression (B) of ABCA1 in THP-1 human macrophages.

A. Relative ABCA1 mRNA abundance increased upon exposure to UK432,097 and this effect was abolished upon pre-incubation with ZM-241385. The data represent the mean and SEM of three independent experiments (n=3).

B. Quantification of Western blot analysis using anti-human ABCA1 specific antibody after 24h incubation of THP-1 macrophages under the following conditions: (1) dimethyl sulfoxide (DMSO) vehicle (solvent control); (2) UK-432,097 (100 nM); (3) ZM-241385 (1 μM) + UK-432,097 (100 nM); (4) CGS-21680 (1 μM); (5) ZM-241385 (1 μM, 1 h) + CGS-21680 (1 μM). β-actin was detected on the same membrane as a loading control. Immunoblot results presented as a graph for ABCA1 protein expression with normalization to β-actin. The data represent the mean and SEM of three independent experiments (n=3).

** - P<0.01 vs. control, ##P<0.01, ### - P<0.001 vs. A2A agonists.

Fig. 4. Effect of UK432,097 on the mRNA level (A) and protein expression (B) of ABCG1 in THP-1 human macrophages.

A. Relative ABCG1 mRNA abundance increased upon exposure to UK432,097 and this effect was abolished upon pre-incubation with ZM-241385. The data represent the mean and SEM of three independent experiments (n=3).

B. Quantification of Western blot analysis using anti-human ABCG1 specific antibody after 24h incubation of THP-1 macrophages under the following conditions: (1) dimethyl sulfoxide (DMSO) vehicle (solvent control); (2) UK-432,097 (100 nM); (3) ZM-241385 (1 μM) + UK-432,097 (100 nM); (4) CGS-21680 (1 μM); (5) ZM-241385 (1 μM, 1 h) + CGS-21680 (1 μM). β-actin was detected on the same membrane as a loading control. Immunoblot results presented as a graph for ABCG1 protein expression with normalization to β-actin. The data represent the mean and SEM of three independent experiments (n=3).

** - P<0.01, *** - P<0.001 vs. control, # - P<0.05, ## - P<0.01, ### - P<0.001 vs. A2A agonists.
**Fig. 5.** Effect of UK432,097 on the mRNA level (A) and protein expression (B) of 27-hydroxylase in THP-1 human macrophages.

**A.** Relative 27-hydroxylase mRNA abundance increased upon exposure to UK432,097 and this effect was abolished upon pre-incubation with ZM-241385. The data represent the mean and SEM of three independent experiments (n=3).

**B.** Quantification of western blot analysis using anti-human 27-hydroxylase specific antibody after 24h incubation of THP-1 macrophages under the following conditions: (1) dimethyl sulfoxide (DMSO) vehicle (solvent control); (2) UK-432,097 (100 nM); (3) ZM-241385 (1μM) + UK-432,097 (100 nM); (4) CGS-21680 (1 μM); (5) ZM-241385 (1 μM, 1 h) + CGS-21680 (1 μM). β-actin was detected on the same membrane as a loading control. Immunoblot results presented as a graph for 27-hydroxylase protein expression with normalization to β-actin. The data represent the mean and SEM of three independent experiments (n=3).

* - P<0.05, ** - P<0.01 vs. control, # - P<0.05, ### - P<0.001 vs. A2A agonists.

**Fig. 6.** Effect of UK432,097 on the mRNA level (A) and protein expression (B) of LXR-α in THP-1 human macrophages.

**A.** Relative LXRα mRNA abundance increased upon exposure to UK432,097 and this effect was abolished with ZM-241385 pre-incubation. The data represent the mean and SEM of three independent experiments (n=3).

**B.** Quantification of western blot analysis using anti-human LXRα specific antibody after 24h incubation of THP-1 macrophages under the conditions described in methods. β-actin was detected on the same membrane as a loading control. Immunoblot results presented as a graph for LXR-α protein expression with normalization to β-actin. The data represent the mean and SEM of three independent experiments (n=3).

* - P<0.05, ** - P<0.01 vs. control, # - P<0.05, ### - P<0.001 vs. A2A agonists.
Methotrexate, a disease modifying anti-rheumatic drug has been shown to reduce cardiovascular events in rheumatoid arthritis patients.\textsuperscript{21,22} Additionally, methotrexate has been shown to inhibit atherogenesis in cholesterol-fed rabbits,\textsuperscript{30} and thus, one of the proposed mechanisms through which methotrexate is cardio-protective is by means of reduction of atherosclerosis. Interestingly it was found that methotrexate increases the extracellular amount of adenosine.\textsuperscript{31} Additionally, our group discovered that methotrexate's athero-protective effect is mediated through an increase of adenosine occupying the A\textsubscript{2a}R.\textsuperscript{32}

Occupation of A\textsubscript{2a}R has been reported to prevent development of atherosclerosis in animal models and to promote cholesterol efflux in cell culture systems.\textsuperscript{33,34} Prior studies have demonstrated that adenosine, acting at the A\textsubscript{2a}R, diminishes foam cell formation in human macrophages and stimulates the expression of ABCA1, ABCG1, 27-hydroxylase, and LXR\textsubscript{Rf}.\textsuperscript{15}

Methotrexate, acting through the release of adenosine and activation of the A\textsubscript{2a}R, elevates RCT proteins such as 27-hydroxylase and ABCA1 levels in human monocytes and macrophages.\textsuperscript{23}

The downside of methotrexate is that it is associated with significant safety issues, such as liver and/or renal dysfunction, that limit its use.\textsuperscript{35,36} Therefore, examining the efficacy of other A\textsubscript{2a}R agonists with a potentially greater safety profile would be beneficial from the standpoint of developing a cardio/athero-protective drug.

Since few A\textsubscript{2a}R agonists have been used successfully in humans, we focus here on UK-432,097, a drug with established safety in human subjects.\textsuperscript{37} The present studies demonstrate that UK-432,097 favorably alters expression of RCT proteins at the level of both message and protein and that this effect is mediated through the A\textsubscript{2a}R. Uregulation of cholesterol efflux via augmentation of RCT proteins explains the beneficial decrease in oxLDL uptake by THP-1 macrophages upon exposure to UK-432,097. This effect may be construed as additional evidence of the anti-atherogenic nature of adenosine receptor agonists because limiting oxLDL accumulation prevents foam cell formation, critical for atherosclerosis progression.

Increased expression of 27-hydroxylase resulting from activation of A\textsubscript{2a}R suggests a means of improving cholesterol outflow. Prior attempts to upregulate 27-hydroxylase enzyme activity in macrophages by adding a variety of hormones were unsuccessful.\textsuperscript{38} Recently, we demonstrated the ability of resveratrol, partially acting through activation of the A\textsubscript{2a}R, to regulate expression of RCT proteins.\textsuperscript{39}

UK-432,097 was initially discovered as an inhaled potent A2a agonist, that stabilizes the receptor upon binding,\textsuperscript{26} and was supposed to serve as an inhaled treatment for inflammatory symptoms of chronic obstructive pulmonary disease. Although, further studies are needed to determine whether the safety profile of UK-432,097 is superior to that of methotrexate, in a recent study examining its clinical efficacy for COPD treatment, UK-432,097 had no safety limitation over the dose range tested in the Phase I clinical study but a Phase II study failed to show efficacy in patients with COPD.\textsuperscript{27,40}

Although A\textsubscript{2a}R agonists have been recognized for their potential clinical use as anti-inflammatory agents,\textsuperscript{41} their application as atheroprotective modulators of lipid metabolism is only recently being explored.\textsuperscript{42} Findings presented in this report provide an example of how adenosine A\textsubscript{2a}R agonists improve cellular cholesterol metabolism and may potentially provide a therapeutic option for reducing the risk of atherosclerosis. This is particularly timely in light of the ongoing cardiovascular inflammation reduction trial (CIRT).\textsuperscript{43} CIRT, funded by the NHLBI, is a randomized, double blind, placebo-controlled clinical trial designed to determine whether low dose methotrexate will reduce recurrent cardiovascular events in 7,000 stable patients with type 2 diabetes or metabolic syndrome who have had a previous MI or have angiographically demonstrated multivessel coronary artery disease. Targeted drugs such as UK-432,097 that specifically homes in on the A\textsubscript{2a}R might be candidates for future cardioprotection trials in coronary artery disease patients.

There are some limitations of our study. We did not explore all possible exposure times of the A\textsubscript{2a}R agonist UK-432,097 on THP-1 cells. However, the times we did use in our study represent typical cell treatment exposure times. Based on the results it is unlikely that the expression levels would be significantly different in the intermediate time.
exposures between 6 and 18 hours. Our study was done using THP-1 monocytes that were differentiated into macrophages, but not polarized to a specific phenotype. We plan future studies using UK-432,097 on classically activated M1 and alternatively activated M2 macrophages.

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