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Cross-Regulation of Carbon Monoxide and the Adenosine A2a Receptor in Macrophages

Arvand Haschemi,^{*‡} Oswald Wagner,[‡] Rodrig Marculescu,[‡] Barbara Wegiel,^{*} Simon C. Robson,^{*} Nicola Gagliani,^{*} David Gallo,^{*} Jiang-Fan Chen,[†] Fritz H. Bach,^{1*} and Leo E. Otterbein^{1,2*}

Adenosine and heme oxygenase-1 (HO-1) exert a wide range of anti-inflammatory and immunomodulatory actions, making them crucial regulatory molecules. Despite the diversity in their modes of action, the similarity of biological effects of adenosine and HO-1 led us to hypothesize a possible interrelationship between them. We assessed a potential role for HO-1 in the ability of adenosine or 5'-N-ethylcarboxamidoadenosine (NECA), a stable adenosine analog, to modify the response of LPS-stimulated macrophages. Adenosine and NECA markedly induced HO-1 and blocked LPS-induced TNF- α production via adenosine A2aR-mediated signaling; blocking of HO-1 by RNA interference abrogated the effects of adenosine and NECA on TNF- α . HO-1 overexpression or exposure to carbon monoxide (CO), a product of HO-1 enzymatic activity, resulted in augmented A2aR mRNA and protein levels in RAW264.7 cells and primary macrophages. The induction of A2aR expression by HO-1 or CO resulted in an increase in the sensitivity to the anti-inflammatory effects of adenosine and NECA, which was lost in macrophages isolated from A2aR-deficient mice. Moreover, a decrease in cAMP levels upon NECA stimulation of naive macrophages was counterbalanced by CO exposure to up-regulate A2aR levels. This implies adenosine receptor isoform switch as a selective modification in macrophage phenotype. Taken together, these data suggest the existence of a positive feedback loop among adenosine, HO-1, CO, and the A2aR in the chronological resolution of the inflammatory response. *The Journal of Immunology*, 2007, 178: 5921–5929.

Adenosine and heme oxygenase-1 (HO-1,³ *hmox-1*) are crucial regulatory molecules in inflammation and immunity. HO-1 is the inducible isoform of the heme oxygenases that are the rate-limiting enzymes in the degradation of heme resulting in the generation of carbon monoxide (CO), iron, and biliverdin. Biliverdin is rapidly converted to bilirubin via biliverdin reductase (1–3). HO-1 is considered a protective, homeostasis-inducing gene, providing a wide range of beneficial effects in numerous models of disease, including endotoxic shock (3–6), ischemia/reperfusion injury (3, 7–9), vascular stenosis (10, 11), and hepatitis (12), to name but a few. The effects of HO-1 are thought to be primarily mediated by one or more of its three products (2, 3, 13).

Adenosine, an endogenous purine nucleoside, is able to bind four distinct G protein-coupled receptors found on the surface of a

diversity of cell types. These four receptors, designated A1, A2a, A2b, and A3, mediate disparate effects in response to adenosine most likely resulting from differences in the receptor-coupled interaction with the diverse family of G proteins at the membrane (14, 15). A source of extracellular adenosine is cytosolic ATP, which may be released by various stimuli that activate macrophages as in inflammation, ischemia, and tissue injury. The released ATP is promptly degraded to adenosine by the extracellular ecto-ATP-ase, ecto-ADP-ase, such as CD39, and ecto-5'-nucleotidase enzymes (14). The effect of adenosine on a given cell is strongly dependent on the adenosine receptor subset present and the resulting intracellular signal transduction cascades that are triggered following ligand binding. In several models of inflammation, the adenosine A2aR mediates anti-inflammatory, whereas the A1R signals for proinflammatory effects of adenosine (16, 17). The individual roles of the other receptor subtypes in immune regulation remain ill defined.

The aim of the present study was to elucidate a possible interrelationship between adenosine and HO-1, two well-known protective molecules (18), in the regulation of inflammation due to the remarkable overlap in their anti-inflammatory properties. This is exemplified by their ability in macrophages to inhibit inducible NO synthase and modulate cytokine production, including inhibition of TNF- α and increasing IL-10 in response to endotoxin (6, 19–24). Additionally, they both down-regulate the adhesion molecules VCAM and E-selectin, but not ICAM in activated endothelial cells (25, 26). Based on the similarities of their actions, we hypothesized that these two highly conserved molecules cooperate in the regulation and resolution of inflammation.

Our data show that adenosine induces HO-1 via the A2aR and that HO-1 in turn selectively alters expression of the A2aR via generation of CO. The increase in the adenosine A2aR expression augments the sensitivity of macrophages toward the

^{*}Transplant and Immunobiology Research Centers, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; [†]Department of Neurology, Boston University School of Medicine, Boston, MA 02118; and [‡]Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria

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¹ F.H.B. and L.E.O. contributed equally to this work.

² Address correspondence and reprint requests to Dr. Leo E. Otterbein, Beth Israel Deaconess Medical Center, 99 Brookline Avenue, Suite 370G, Boston, MA 02215. E-mail address: lotterbe@bidmc.harvard.edu

³ Abbreviations used in this paper: HO-1, heme oxygenase-1; 2-CADO, 2-chloradenosine; Ad-EGFP, adenovirus for enhanced GFP; Ad-HO-1, adenovirus for HO-1; BMDM, bone marrow-derived macrophage; CHO, Chinese hamster ovary; EGFP, enhanced GFP; EHNA, *erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride*; HA, hemagglutinin; NECA, 5'-N-ethylcarboxamidoadenosine; RT, room temperature; siRNA, small interfering RNA.

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anti-inflammatory effect of adenosine. Taken together, our data support the concept of a regulatory loop existing between adenosine signaling and HO-1 induction, enabling a system whereby macrophages can actively participate in the resolution of inflammation and re-establish homeostasis.

Materials and Methods

Chemicals

LPS (*Escherichia coli* B055:02), adenosine, 5'-*N*-ethylcarboxamidoadenosine (NECA), hemin, biliverdin, and 2-chloradenosine (2-CADO) were from Sigma-Aldrich. ATL146e was generously provided by Adenosine Therapeutics.

Cell culture and animals

The mouse macrophage cell line RAW264.7 (American Type Culture Collection), the stable transfected cell line HO-1 RAW264.7, the control cell line NEO RAW264.7 (6), and Chinese hamster ovary (CHO) cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% heat-inactivated FBS and 50 $\mu\text{g}/\text{ml}$ gentamicin in an atmosphere with 5% CO_2 at 37°C. Bone marrow-derived macrophages (BMDM) were differentiated by M-CSF (2 ng/ml), as previously described (27). Male C57BL/6 wild-type and adenosine A2aR-deficient mice were generated, as previously described (28).

CO exposure

Cells were exposed to CO, using a bioactive gas-controlling system custom designed and built by Biospherix. To achieve a concentration of 250 ppm, CO (Lifegas) was mixed with 5% CO_2 /20.8% O_2 /74% N_2 and controlled by Watflow Anafaze software.

SDS-PAGE and Western blotting

Total cell lysates were made by harvesting cells in 1% SDS, 10 mM Tris-HCl (pH 7.4) containing Complete protease inhibitors (Roche). Protein samples were heat denatured at 95°C for 5 min, sonicated, and centrifuged at $10,000 \times g$ for 10 min. Protein concentrations in the supernatants were determined using the bicinchoninic acid assay (Pierce). Cell lysates in 1× sample buffer containing 2-ME were boiled for 5 min. Samples were resolved on 4–12% polyacrylamide gels (Invitrogen Life Technologies) and transferred to polyvinylidene difluoride (Bio-Rad). Loading of protein samples was monitored by staining with Ponceau Red. After overnight blocking with 5% nonfat dry milk in 0.05% Tween 20 TBS, blots were incubated with Abs against HO-1 (SPA-896; StressGen Biotechnologies), adenosine A2aR (clone 7F6-G5-A2; Upstate Biotechnology), or influenza hemagglutinin (HA) tag (ab9110; Abcam). Ab-Ag complexes were visualized using HRP-conjugated Ab against rabbit or mouse IgG and SuperSignal West Pico (HO-1, HA) or Femto (A2aR) chemiluminescent substrate, per manufacturer's instructions (Pierce). To confirm equal loading, blots were re-probed with mouse mAb targeting GAPDH (Abcam) or rabbit polyclonal anti- β -actin (Cell Signaling Technology). ImageJ software (National Institutes of Health, version 1.37) was used for quantitative densitometric analysis of gel band intensities. To control for the specificity of the A2aR Ab used, we treated cell lysates with *N*-glycosidase F, which results in a characteristic decrease in the molecular mass of A2aR protein (data not shown) (29).

Transient transfection of CHO cells

CHO cells were transiently transfected with pcDNA3.1-HA, pcDNA3.1-HA-A1R, or pcDNA3.1-HA-A2aR by using Nucleofector technology. The electroporations were performed using Nucleofector kit T, according to the manufacturer's instructions (Amaxa Biosystems). The constructs expressing adenosine A1R as well as adenosine A2aR were obtained from UMR cDNA Resource Center (www.cdna.org), and inserts were verified by directed restriction digest.

Measurement of TNF- α , IL-12, and IL-10 in culture supernatants

Cytokine secretion was measured using mouse TNF- α /TNFSF1A, IL-12p70, and IL-10 Quantikine ELISA kits purchased from R&D Systems, according to manufacturer's directions.

Real-time PCR

Total RNA (1 μg), isolated with RNeasy (Qiagen), was reverse transcribed into cDNA using the Moloney murine leukemia virus enzyme (Applied

Biosystems) and random hexamer primers (1 $\mu\text{g}/\mu\text{g}$ total RNA). The reaction mixture was incubated at 25°C for 10 min, followed by 30 min at 48°C and 5 min at 95°C. All PCR were performed using the TaqMan Universal PCR kit (Applied Biosystems). Reactions were set up in a total volume of 25 μl by using 2× Taqman Universal PCR Master Mix (Applied Biosystems). Each cDNA sample was analyzed in duplicate in the presence of 1× commercially available assay-on-demand primers together with 6-carboxyfluorescein-labeled fluorogenic probes (Applied Biosystems) designed for each target gene. GAPDH expression of each sample was performed in parallel to standardize the amount of sample cDNA added to the reaction. Using a Mx3000P QPCR System (Stratagene), PCR cycling conditions were as follows: 2 min start at 50°C, denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 60 s. Sequence Detector Software (Stratagene) was used to extract the PCR data, which were then exported to Excel (Microsoft) for further analysis. Relative quantitation of adenosine A2aR and A2bR expression was analyzed based upon a comparative cycle cross-threshold method, normalized to GAPDH.

cAMP determination

cAMP was measured in RAW264.7 cells using a Direct Cyclic AMP Enzyme immunoassay kit (Assay Designs), according to the manufacturer's instructions. Cells were grown on 100-mm culture plates and lysed in 350 μl of 0.1 M HCl.

RNA interference and adenoviral infection

Pre-designed small interfering RNA (siRNA) against mouse HO-1 (siRNA ID: 158979) and control siRNA were obtained from Ambion. RAW264.7 cells were transfected in six-well format with either 50 nmol HO-1 or a control siRNA in OptiMEM using Lipofectamine 2000 (according to the manufacturer's instructions; Invitrogen Life Technologies). Twenty-four hours after transfection, cells were then used for further experimentation. Infection of RAW264.7 cell with adenovirus (at 30 multiplicity of infection) expressing either HO-1 or enhanced GFP (EGFP) was performed, as previously described (30).

Immunofluorescence

RAW264.7 macrophages were grown on glass coverslips. Briefly, for immunofluorescence microscopy, coverslips were washed twice in 1× PBS and exposed to prechilled methanol at -20°C for 2 min. Methanol was removed quickly, and the coverslips were washed three times with 1× PBS. Fixed cells were blocked overnight at 4°C with blocking solution (2% BSA in PBS). Incubation with A2aR mAb (1:300; clone 7F6-G5-A2; Upstate Biotechnology) was performed at room temperature (RT) for 2 h, followed by three washing steps with 1× PBS for 20 min. Alexa-594-conjugated goat anti-mouse Ab (Molecular Probes) (1:1000) was added for 2 h at RT in the dark. Nuclei were visualized with Hoechst 33258 (Sigma-Aldrich). Slides were dried overnight at RT in the dark. Specimens were then examined using a Zeiss Axiovert Microscope (Carl Zeiss Optics).

Statistical analysis

Data are presented as mean \pm SD. Comparisons were performed with ANOVA, followed by the Bonferroni test. Differences were considered significant at $p < 0.05$.

Results

Adenosine and NECA induce HO-1 expression in macrophages via A2aR stimulation

Administration of NECA, a stable adenosine analog, induced a dose-dependent increase in HO-1 expression in RAW264.7 cells, as measured by Western blots after 8 h of incubation (Fig. 1A). HO-1 induction was observed at concentrations of 1 μM and peaked at 10 μM , with no additional induction at 50 μM . We thus used 10 μM for subsequent studies. A kinetic analysis of HO-1 induction following administration of NECA (10 μM) to RAW264.7 cells showed an initial increase of HO-1 protein at 4 h with maximal expression peaking at 6–12 h and declining to baseline by 24 h (Fig. 1B).

To confirm the selective effect of NECA, we incubated RAW264.7 cells separately with the following agents: adenosine/erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) (100 μM /100 μM), 2-CADO (A1R agonist) 10 μM , or the selective A2aR agonist ATL146e (10 μM). All agonists led to a

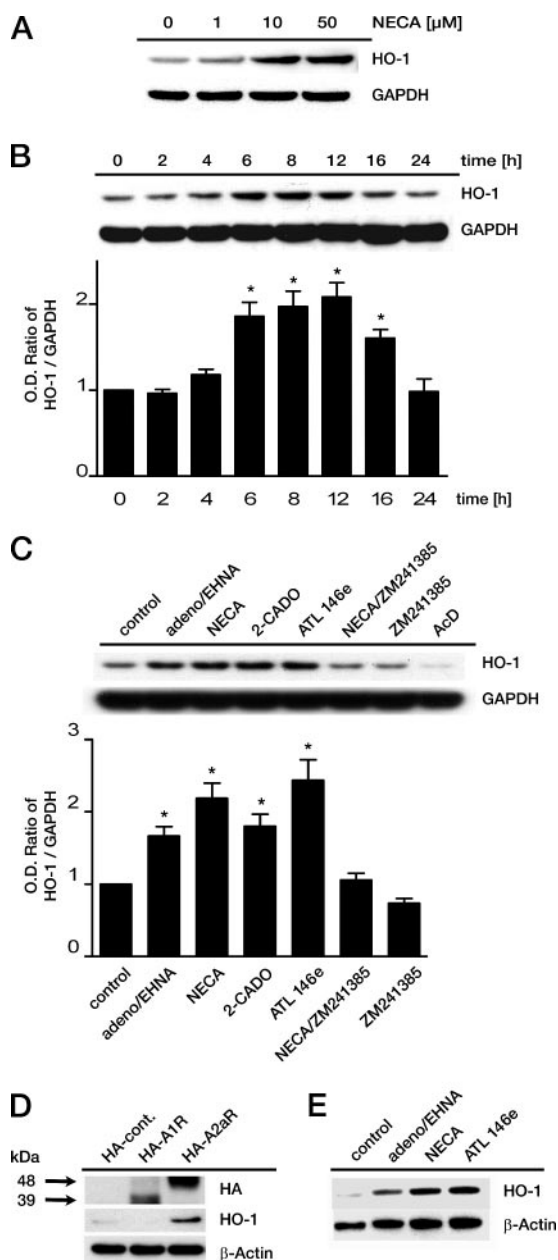


FIGURE 1. Up-regulation of HO-1 protein in RAW264.7 mouse macrophages stimulated by adenosine and analogues. *A*, Western blot of cell extracts from RAW264.7 cells incubated with 0, 1, 10, or 50 μ M NECA for 8 h was analyzed for HO-1 protein. GAPDH was used as a loading control. *B*, Time-dependent up-regulation of HO-1 protein by 10 μ M NECA. Western blots and corresponding densitometric analysis are shown. *C*, Western blot and corresponding densitometric analyses are shown for HO-1 expression in RAW264.7 macrophages exposed to adenosine/EHNA (100 μ M/100 μ M), NECA (10 μ M), 2-CADO (10 μ M), ATL146e (10 μ M), NECA/ZM241385 (10 μ M/10 μ M), or ZM241385 (10 μ M) for 8 h (upper panel). *D*, CHO cells were transfected with either control plasmid pcDNA3.1-HA-tagged, pcDNA3.1-HA-A1R, or pcDNA3.1-HA-A2aR, and 24 h posttransfection cells were stimulated with 10 μ M NECA for 8 h. Cell lysates were subjected to immunoblotting and probed for HA-tagged recombinant proteins. HO-1 expression was evaluated by Western blot. β -Actin was used as a loading control. *E*, U937 cells were incubated with adenosine/EHNA (100 μ M/100 μ M), NECA (10 μ M), and ATL146e (10 μ M) for 6 h and subjected to Western blot analysis for HO-1. β -Actin was used to show equal loading. All Western blots are representative of at least three independent experiments.

significant increase in HO-1 protein expression after 8 h of stimulation that was comparable to the effects observed with NECA (Fig. 1C). Adenosine in the absence of the adenosine deaminase inhibitor (EHNA) showed relatively weak HO-1 induction compared with NECA, which is most likely due to the rapid degradation of adenosine (data not shown). Western blots revealed that ATL146e is as potent as NECA in inducing HO-1 expression, whereas administration of ZM241385, a selective antagonist of A2aR, completely blocked NECA-dependent up-regulation of HO-1. These findings suggest that the A2aR is critical in the up-regulation of HO-1 by adenosine (Fig. 1C).

To dissect the adenosine receptor signaling and to confirm the importance of the A2aR in mediating the signal that leads to induction of HO-1, we used CHO cells that do not express endogenous adenosine receptors (31). As shown in Fig. 1D, we individually introduced a 5'-HA-tagged A1R, a 5'-HA-tagged A2aR or a control plasmid HA-tagged pcDNA3.1 into CHO cells (see *Materials and Methods*). Recombinant receptor expression was detected by a HA-reactive Ab. As shown in Fig. 1D, we observed expression of each receptor subtype in CHO cells with the expected differences in molecular weights of HA recombinants due to the different sizes of each respective receptor isoform. We then stimulated these cells with NECA (10 μ M) and assessed HO-1 protein expression by Western blot. Importantly, HO-1 induction was observed in those cells expressing the A2aR subtype, but not in cells presenting the adenosine A1R (Fig. 1D).

Treatment of the U937 human monocyte cell line with adenosine/EHNA (100 μ M/100 μ M), NECA (10 μ M), or the selective A2aR agonist ATL146e (10 μ M) resulted in a significant increase in HO-1 protein expression after 6 h of stimulation that was comparable to the effects observed in the mouse macrophage RAW264.7 cells (Fig. 1E). These data demonstrate that up-regulation of HO-1 by an A2aR-mediated mechanism is not limited to murine cells.

Inhibition of the LPS-mediated TNF- α release by adenosine and NECA is dependent on HO-1 expression

Adenosine and NECA have the ability to suppress TNF- α production in macrophages exposed to LPS (17, 23, 24). We used this model to evaluate early and suboptimal anti-inflammatory effects of adenosine and to explore a possible relationship between adenosine effects and HO-1 expression in the suppression of inflammation. To validate the model and verify the anti-inflammatory properties of adenosine, we incubated RAW264.7 cells with 100 μ M adenosine, stimulated them with 1 ng/ml LPS, and analyzed TNF- α secretion into the supernatant over 5 h (Fig. 2A). As expected, adenosine administration blocked TNF- α secretion. This effect became significant after 2–4 h poststimulation, and therefore we used the 4-h time point for subsequent experiments. Early production of TNF- α is regulated at the transcriptional level and thus not expected to be modulated by adenosine. Adenosine has no effect on TNF- α mRNA levels, as recent publications have reported (32) and own data suggest (data not shown). To unearth a possible role for HO-1 in the inhibition of TNF- α release by adenosine, we blocked expression of HO-1 using RNA interference. RAW264.7 murine macrophages, transiently expressing siRNA targeting HO-1 or control siRNA constructs, were treated with adenosine or NECA 30 min before LPS stimulation. TNF- α levels in cell supernatants were measured 4 h after LPS administration (Fig. 2B). The ability of adenosine or NECA to block TNF- α generation was lost in those cells in which basal HO-1 expression was blocked. These findings implicate HO-1 as critical in the ability of adenosine to exert its anti-inflammatory effects.

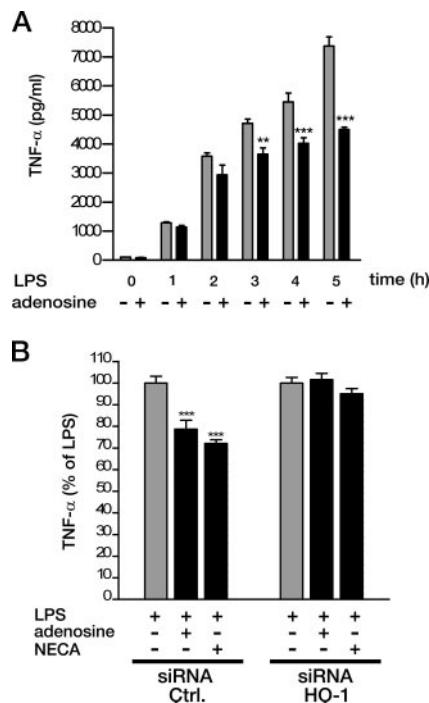


FIGURE 2. Inhibition of HO-1 leads to loss of the inhibitory effect of adenosine or NECA on LPS-induced TNF- α production in RAW264.7 macrophages. **A**, Kinetic analysis of LPS (1 ng/ml)-induced TNF- α secretion in the presence (■) or absence (□) of 100 μ M adenosine measured by ELISA. Graph is representative of three independent experiments. **B**, RAW264.7 cells were transiently transfected with 50 nmol HO-1-siRNA or Ctrl-siRNA for 24–36 h. The effect of adenosine (100 μ M) or NECA (10 μ M) on TNF- α production was monitored after LPS administration (1 ng/ml, 4 h). Data were normalized to LPS controls. $n = 3$; **, $p < 0.01$; ***, $p < 0.001$.

Overexpression of HO-1 increases the inhibitory effect of adenosine and NECA on the TNF- α response

Having shown that HO-1 is involved in the effects observed with adenosine and NECA, we tested whether increased HO-1 expression would enhance the effect of adenosine. To test this hypothesis, we generated stably transfected RAW264.7 cells that overexpress HO-1 as well as wild-type RAW264.7 cells infected with a recombinant adenovirus expressing HO-1 (Fig. 3A). These cells and their appropriate controls were separately treated with NECA or adenosine, followed by LPS stimulation. Stable HO-1 overexpression in macrophages boosted the ability of adenosine and NECA to block TNF- α : there was a 46 and 39% increase in efficacy, respectively, compared with control cells ($19.87 \pm 3.61\%$ in control cells vs $36.13 \pm 2.99\%$ in HO-1 RAW264.7 cells with adenosine and 26.34 ± 4.98 to $42.53 \pm 3.14\%$ (NEO vs HO-1, respectively) inhibition with NECA treatment; Fig. 3B). Moreover, this boost in the ability of NECA to block LPS-induced TNF- α production becomes more apparent when comparing the dose-response curve of NECA in the two respective cell lines (Fig. 3C).

This augmentation in adenosine and NECA-mediated TNF- α inhibition in stably transfected HO-1 cells was verified using RAW264.7 cells infected with a recombinant adenovirus to transiently overexpress HO-1 (adenovirus for HO-1 (Ad-HO-1)). Ad-HO-1 cells showed a 2-fold greater effect of NECA on the inhibition of TNF- α production compared with control adenovirus for EGFP (Ad-EGFP)-infected cells, excluding an artifact of the HO-1 stably transfected cell line (Fig. 3D). More dramatic effects were observed by decreasing NECA concentrations. The response of

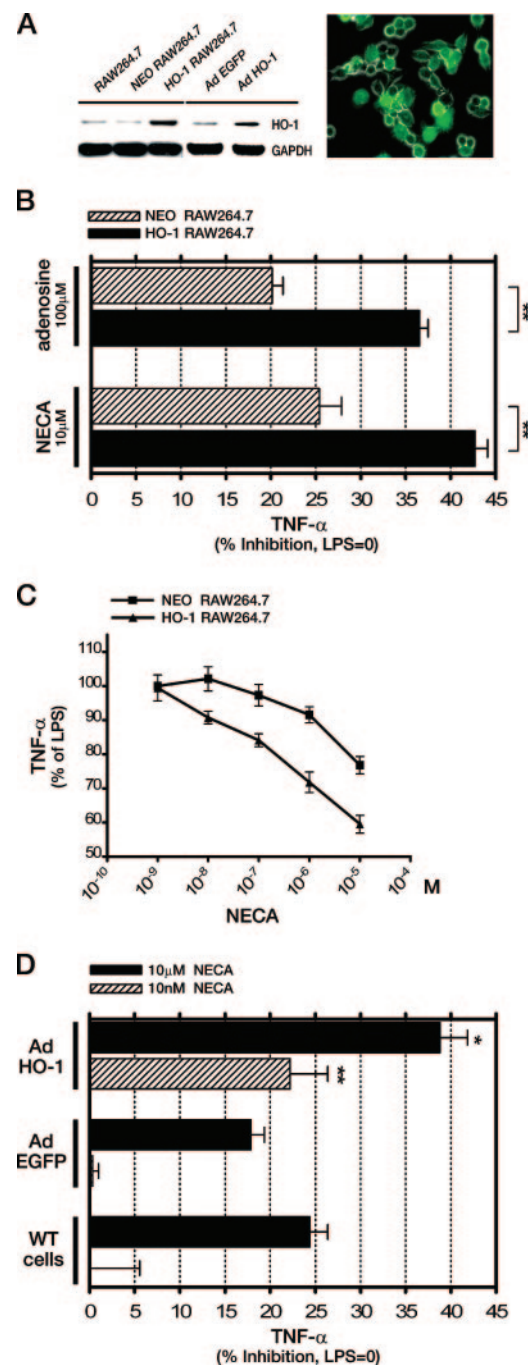


FIGURE 3. HO-1 overexpression sensitizes RAW264.7 macrophages to the anti-inflammatory effect of adenosine and NECA. **A**, RAW264.7 cells stably transfected with NEO (empty vector) or HO-1 expression vectors (see Ref. 6 for method) or infected with Ad-HO-1 or Ad-EGFP as control. Immunoblot shows HO-1 protein levels in RAW264.7, HO-1 RAW264.7, NEO RAW264.7, and cells infected either with HO-1 or EGFP. Efficiency of infection was further assessed by fluorescence microscopy of Ad-EGFP cells. **B**, Inhibition of TNF- α production (percentage of inhibition compared with LPS treated) by 100 μ M adenosine or 10 μ M NECA after 4 h of LPS (1 ng/ml) stimulation in HO-1 stable transfected RAW264.7 (HO-1 RAW264.7) and control cell line (NEO RAW264.7). **C**, Dose response of NECA on TNF- α production in LPS-activated HO-1 RAW264.7 compared with NEO RAW264.7 cells. Note the difference in sensitivity between HO-1 and control cells in terms of the NECA concentration used, as follows: 10^{-8} M ($p < 0.05$), 10^{-7} M, 10^{-6} M, and 10^{-5} M (all $p < 0.01$ vs controls). **D**, Effect of 10 μ M and 10 nM NECA in adenoviral infected (HO-1 and EGFP) and wild-type macrophages treated with LPS. TNF- α results are given as percentage of inhibition. Each group was normalized separately to exclusively illustrate the adenosine or NECA effect. $n = 3-4$; *, $p < 0.05$; **, $p < 0.01$.

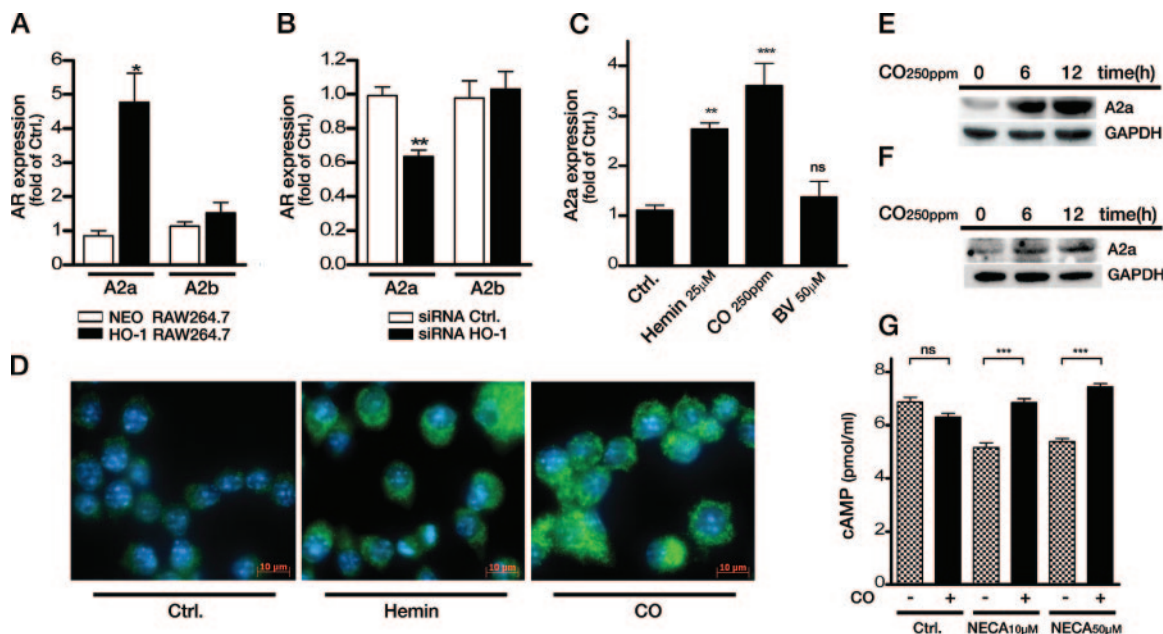


FIGURE 4. Induction of the functional adenosine A2aR by the HO-1/CO system. *A* and *B*, Adenosine receptor (AR) A2a and A2b mRNA levels were measured by real-time PCR in RAW264.7 cells stably transfected with HO-1 or control NEO (*A*) and siRNA HO-1-transfected RAW264.7 macrophages (*B*). The $\Delta\Delta CT$ (cycle threshold) values were calculated by normalization with GAPDH expression. *C*, Adenosine A2aR expression in RAW264.7 cells following treatment with either 25 μ M hemin, CO (250 ppm), or 50 μ M biliverdin for 12 h. Experiments were performed in triplicate, and fold changes were calculated, as described above. *D*, Immunofluorescence microscopy images were obtained from MeOH-fixed cells after treatment with hemin (25 μ M) or CO (250 ppm) for 12 h. Nuclei are stained in blue with Hoechst 33258 and A2aR in green. *E* and *F*, A2aR expression by Western blot from RAW264.7 cells (*E*) or bone marrow-derived macrophages (*F*) treated with 250 ppm CO for 0, 6, or 12 h; GAPDH indicates equal loading. Blots are representative of three independent experiments. *G*, cAMP production was measured after 30 min of 10 and 50 μ M NECA incubation in either control (air) or CO (250 ppm, 12 h)-treated RAW264.7 cells. $n = 3$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Ad-HO-1 macrophages to a 1000-fold lower (10 nM) concentration of NECA was as effective as the concentration (10 μ M) required in wild-type and Ad-EGFP cells to achieve the same inhibition of TNF- α production (Fig. 3*D*). In both control infected and noninfected wild-type cells, a concentration of 10 nM NECA had no effect on LPS-induced TNF- α levels: NECA was effective at blocking TNF- α production in the nanomolar range only in those cells with high HO-1 expression.

HO-1 increases expression of the adenosine A2aR via CO signaling

To elucidate the mechanism of the observed relationship between adenosine and HO-1, we examined adenosine A2aR and A2bR expression in RAW264.7 macrophages in the presence and absence of HO-1. As shown in Fig. 4, *A* and *B*, we assessed the mRNA expression of the A2aR and A2bR in cells stably overexpressing HO-1 as well as in untreated cells transfected with HO-1 siRNA. In cultured HO-1-overexpressing RAW264.7 cells, we observed a significant increase in adenosine A2aR mRNA expression as compared with control cells; A2aR expression was significantly decreased after transient transfection with HO-1 siRNA. A2bR expression remained unchanged in both HO-1 RAW264.7 and HO-1 siRNA compared with NEO-RAW264.7 and control siRNA-expressing cells. Treatment with hemin (25 μ M) to induce HO-1 showed a comparable effect on A2aR expression as observed in the cell line stably overexpressing HO-1, thereby again excluding an artifact of the HO-1 stably transfected cell line in this regard (Fig. 4*C*).

To determine a potential mechanism by which HO-1 regulated induction of the adenosine A2aR mRNA, we treated RAW264.7 macrophage cells with either CO or biliverdin at concentrations that have widely been used in RAW264.7 macrophages (6, 33)

(Fig. 4*C*). Treatment with CO (250 ppm), but not biliverdin (50 μ M), induced A2aR mRNA similarly to that observed with increased HO-1 expression. These increases were also observed by immunofluorescence staining for the A2aR protein (Fig. 4*D*). RAW264.7 cells treated with CO (250 ppm) for 6 and 12 h show a clear and significant increase in A2aR by Western blot analysis vs air-exposed cells (Fig. 4*E*). We further confirmed CO (250 ppm)-induced A2aR expression in primary murine BMDM after 6 and 12 h of incubation (Fig. 4*F*). CO-induced A2aR expression was observed at both the mRNA and protein levels.

CO alters the cAMP response of naive macrophages to NECA

To explore the functionality of the increased A2aR expression induced by CO, we incubated RAW264.7 macrophages in the presence and absence of CO (250 ppm) for 12 h, followed by subsequent NECA stimulation for 30 min, and measured cAMP production, which is a well-described intracellular second messenger generated in response to A2aR binding. Of note, in non-CO-treated cells, stimulation with NECA led to a significant decrease in cAMP production, possibly due to inhibition of adenylate cyclase activity through NECA-induced activation of predominantly G_i protein-coupled receptors (Fig. 4*G*). CO exposure of macrophages counterbalanced NECA-induced inhibition in cAMP production as it occurred in naive cells. CO significantly ($p < 0.001$) changes the phenotype of the macrophage to adenosine presumably by its up-regulation of the A2aR.

CO potentiates the anti-inflammatory response to NECA in wild-type, but not in A2aR-deficient primary macrophages

BMDM from A2aR-deficient and wild-type mice were assessed for the ability of NECA to suppress TNF- α release in response to

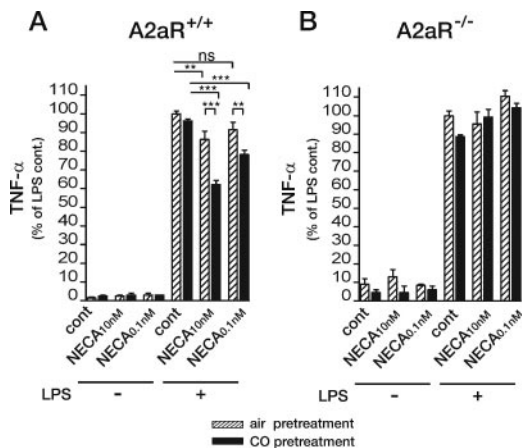


FIGURE 5. CO enhances NECA-induced inhibition in TNF- α production in primary macrophages by an A2aR-dependent mechanism. Primary BMDM from wild-type mice (A) or A2aR-deficient mice (B), treated with either CO (250 ppm) or air for 12 h, were subsequently stimulated with NECA at 10 or 0.1 nM 30 min before LPS (1 ng/ml) challenge. Four hours after LPS, TNF- α levels were analyzed by ELISA. **, $p < 0.01$; ***, $p < 0.001$.

LPS. We first studied wild-type BMDM cultured in air or in a CO environment. After exposure to CO or air, BMDM were stimulated with NECA at either 0.1 or 10 nM and treated with LPS for 4 h.

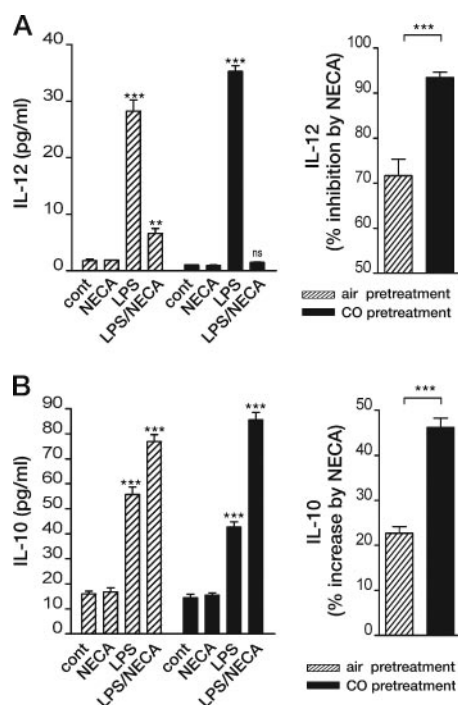


FIGURE 6. Effect of CO on NECA-mediated regulation of IL-12 and IL-10 in LPS-stimulated primary macrophages. Primary BMDM, treated with either CO (250 ppm) or air for 12 h, were subsequently stimulated with NECA (10 nM) 30 min before LPS challenge. A, IL-12 expression after 12-h LPS in the presence and absence of NECA was analyzed in medium by ELISA. Left panel, Illustrates results from three independent experiments. Right panel, Shows the effects of NECA in air- and CO-pretreated cells, calculated as percentage of inhibition ($n = 3$). B, IL-10 expression after 16-h LPS treatment in the presence and absence of NECA and CO was analyzed in medium by ELISA. Left panel, Illustrates results from three independent experiments. Right panel, Shows the effects of NECA in air- and CO-pretreated cells, calculated as percentage of increase ($n = 3$). Data were normalized to LPS controls. Note that in the same cells, NECA block IL-12 and augment IL-10. **, $p < 0.01$; ***, $p < 0.001$.

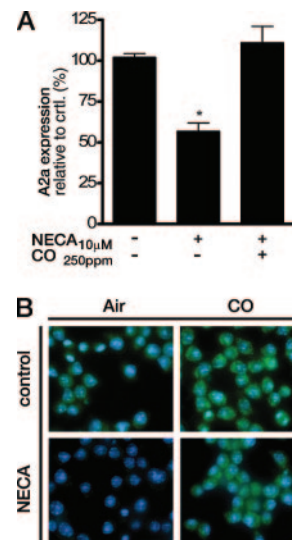


FIGURE 7. CO prevents stimulation-dependent down-regulation of A2aR. A, NECA prevents down-regulation of the adenosine A2aR was measured after 18 h of stimulation by real-time PCR in RAW264.7 cells. $\Delta\Delta CT$ (cycle threshold) values were calculated by normalization to GAPDH expression, and results are displayed as percentage of untreated cells (100%). $n = 3$; *, $p < 0.01$. B, Comparison of immunofluorescence staining of adenosine A2aR after 18 h in the presence or absence of NECA (10 μM). Cells were exposed to either air or CO (250 ppm).

Wild-type macrophages showed the expected reduction in TNF- α production when treated with 10 nM NECA, whereas 0.1 nM NECA showed no significant effect (Fig. 5A). A more dramatic inhibition in TNF- α production was observed at a dose of 10 nM NECA in wild-type cells pretreated with CO for 12 h vs air-treated cells (38% increase of NECA effect in CO vs air-treated cells). Moreover, the subnanomolar dose of NECA (0.1 nM) in CO-exposed macrophages was as effective in blocking TNF- α as was the 10 nM dose in air-treated cells. No reduction in TNF- α was observed after NECA treatment in A2aR-deficient BMDM, suggesting that A2aR mediated the observed NECA action in this model (Fig. 5B). The potentiation effects observed in wild-type cells exposed to CO and treated with NECA were absent in the A2aR-deficient cells, thus corroborating the proposed mechanism of CO action. Of note, untreated A2aR-deficient macrophages expressed higher basal levels of TNF- α vs wild-type controls.

Analysis of IL-10 and IL-12 secretion

To further confirm the proposed mechanism, we next studied IL-10 and IL-12 expression, which are both known to be regulated by A2aR signaling in macrophages (23, 24, 34–36). We cultured BMDM in air or in a CO environment for 12 h and then subjected these cells to LPS for 12 h (IL-12) or 16 h (IL-10). IL-12 production was significantly inhibited by NECA in air-treated cells (Fig. 6A, left panel). However, when NECA was combined with CO, we observed enhanced inhibition in IL-12 expression (Fig. 6A, right panel). Conversely, IL-10 expression in these same cells was augmented in BMDM treated with LPS and NECA compared with cells treated with LPS alone (Fig. 6B, left panel). Again, detailed comparison of the LPS/NECA-treated group in CO- and air-exposed cells showed further augmentation of IL-10 expression in the combination NECA plus CO-treated cells (Fig. 6B, right panel). These data further substantiate the proposed interrelationship between CO and the A2aR, and demonstrate that the mechanism is not limited to

TNF- α regulation, rather imparting a selective immune-regulatory function.

CO blocks NECA-induced down-regulation of the A2aR

Down-regulation of the adenosine A2aR by stimulation with NECA is a well-described phenomena (Fig. 7A) (37, 38). We tested the hypothesis that administration of CO would counteract this decrease in adenosine A2aR expression. Administration of 10 μ M NECA to RAW264.7 macrophages led to an expected decrease in A2aR expression of $45.06 \pm 4.85\%$ as measured by mRNA quantitation ($p < 0.01$), which is in agreement with the findings of Palmer et al. (38). CO exposure in combination with NECA prevented the decrease in A2aR mRNA and even slightly increased protein expression over the indicated time of exposure (Fig. 7). Additional exposure times and concentrations of CO were not tested.

Discussion

Inflammation is a hallmark of many human diseases. There exists a delicate balance by which inflammation peaks and is then resolved. Cells and organisms have evolved to incorporate redundant protective or homeostatic molecules into their armamentarium to maintain and re-establish normal function endogenously. HO-1 and adenosine are two such molecules. Although there are a plethora of protective genes, including the anti-oxidant enzymes, the antiapoptotic genes, and the heat shock protein family, there is little understanding about whether these molecules interrelate functionally to mediate their effects or whether they exist to allow multiple backup strategies by which survival is ensured. The exceptions to this statement are the observations that some anti-inflammatory genes and molecules function only in the presence of HO-1 (3). HO-1 has been proposed to act as a funnel or conduit for many other signaling molecules such as IL-10, PGs, or statins. If HO-1 is inhibited, the function of molecules such as those mentioned above is lost (3, 21, 22). In this study, we provide data in support of a functional interrelationship between adenosine and HO-1. To allow adenosine to become anti-inflammatory, HO-1 must be expressed. HO-1 expression in turn elicits anti-inflammatory effects of its own. However, the situation is more complex because adenosine and HO-1 function diversely. In this study, we show that adenosine is a potent inducer of HO-1 in macrophages and that induction of HO-1 in turn increases the expression of the A2aR via CO generation. We provide data in this work that most likely explain how adenosine, in macrophages, is able to facilitate strong anti-inflammatory effects in a delayed fashion. These findings are somewhat different from the proposed funnel. HO-1 in this instance would be implicated as having a regulatory function that influences the protective response to the molecule, in this case adenosine. HO-1, in summary, ensures an active regulatory system to modulate the expression and function of many protective molecules.

Our findings strongly support the concept that adenosine functions via the A2aR to induce HO-1, which, through the generation of CO, results in a subsequent increase in A2aR expression. Selective stimulation of the adenosine A2aR with the specific receptor agonist ATL146e leads to rapid induction of HO-1 in macrophages. Induction of HO-1 \times 2-CADO in RAW264.7 cells also seems to be mediated by A2aR because CHO cells expressing the A1R show no induction of HO-1, suggesting that 2-CADO is able to exert A2aR-mediated effects, which is in agreement with findings by others (39, 40). The mechanism by which the A2aR signaling increases HO-1 remains unclear, but is most likely regulated by an early burst of reactive oxygen species and an increase in

NRF-2, both of which occur in response to NECA (our unpublished observations) and are known to regulate HO-1 gene expression (2, 41, 42).

The aim of the present study was to investigate the cellular behavior of bacterial endotoxin-exposed macrophages in response to adenosine, rather than exploring the molecular mechanism of a given receptor isoform, which has already been studied extensively. We provide evidence that adenosine loses its capacity to regulate the natural immune response in the absence of HO-1, yet gains effectiveness ($\sim 40\%$ in blocking TNF- α production) when HO-1 and CO levels are increased, suggesting an important communication between these molecules in macrophage regulation and function. HO-1 and CO, in addition to their pleiotropic anti-inflammatory effects, increase expression of the A2aR and thereby amplify sensitivity to adenosine to initiate resolution of inflammation. This mechanism holds true in primary cells, as shown by the studies in BMDM from wild-type and A2aR-deficient mice. Similar effects were also observed in human cells. More generally, the alteration in adenosine action, mediated in macrophages by CO, may imply a change in the activation state of these immune-competent cells and therefore on the general outcome of inflammation. Our data on cAMP production by NECA stimulation in naive macrophages compared with cells pre-exposed to CO support this concept of a phenotype switch, because cAMP is known to possess strong anti-inflammatory properties. Moreover, the ability of LPS itself to induce A2aR in these macrophages (16) further supports the proposed hypothesis of A2aR up-regulation as a time-regulated switch from a pro- to an anti-inflammatory phenotype in macrophages. A possible mechanism by which CO triggers these effects may involve cytochrome *c* oxidase binding in the mitochondria of RAW264.7 cells. Recent reports by others and us show that CO increases mitochondrial reactive oxygen species by interfering with electron transport in a manner similar to events that occur under hypoxic conditions (43–45). Sitkovsky and Ohta (46) and Olah and Caldwell (47) report that hypoxia created at sites of inflammation may function as a stop signal for surrounding cells and tissue so as to allow the resolution of inflammation in part mediated by adenosine signaling. Hypoxia itself ensures that adenosine and CO concentrations will increase by the inhibition of adenosine kinase and induction of HO-1 expression regulated by hypoxia-inducible factor-1 α (43, 48).

We posit a system of interdependence and bidirectional regulation among adenosine, HO-1, CO, and the A2aR that leads to the resolution of the inflammatory response, thereby ultimately re-establishing homeostasis. Critical in this model are the kinetics. First, the naive macrophages are recruited and activated at the site of inflammation accompanied by the local generation of adenosine. Binding of adenosine to A1R, a G_i -coupled receptor, decreases cAMP, resulting in a proinflammatory response. The proinflammatory host defense response is essential and must continue for a given time period to be effective. Therefore, adenosine should not subvert this essential function of the macrophage too quickly, but rather to increase the required innate response of the macrophage. Second, early ligation of the A2aR by adenosine is not sufficient to block the initiating proinflammatory response, but is able to signal the macrophage to up-regulate HO-1. HO-1 and ultimately endogenously generated CO lead to an enhanced anti-inflammatory response both via the direct action of CO and via the up-regulation of A2aR expression, which occurs significantly later in time (>6 h) because HO-1 is regulated transcriptionally. Finally, adenosine via the A2aR that is present at a higher expression level directs its ligand-mediated effects toward increasing cAMP production and

an anti-inflammatory phenotype to re-establish homeostasis. Hypoxia, occurring at the site of most inflammatory situations, may also use a similar mechanism and in fact contribute to the regulation of HO-1 and adenosine. CO generated by increased HO-1 protein may further mimic hypoxia by generating reactive oxygen species or binding to heme in the cellular O₂ sensors (2, 43). Unpublished data from our laboratory suggest that CO can increase expression of hypoxia-inducible factor-1 α , a hypoxia-dependent transcription factor.

Recent studies in the brain by Lin et al. (49) and Ohata et al. (50) support a bidirectional relationship between adenosine and HO-1, which contributes to central cardiovascular regulation. Blockade of either the A_{2A}R or HO-1 resulted in abrogation of the neurophysiologic response to adenosine and HO-1, suggesting that the communication among adenosine, A_{2A}R, and CO outlined in this work is not limited to the immune system, but may also impart crucial regulatory functions in the brain.

In summary, adenosine exerts both proinflammatory and anti-inflammatory effects. We have focused primarily on the interaction of HO-1 expression and the anti-inflammatory effects of adenosine. The ability of HO-1 and adenosine to communicate as well as to initiate the resolution of the inflammatory response may have implications in many aspects of medicine. Treatment of inflammatory diseases today is largely based on interrupting the action of mediators leading to pathophysiology. An alternative approach is to develop novel therapeutics with endogenous or natural mediators essential for the resolution of inflammation, a relatively new area of inflammation research. CO and adenosine A_{2A}R signaling can be exploited as such therapeutic modalities in a wide spectrum of disorders such as sepsis, chronic inflammation, and organ transplantation, to name but a few.

Disclosures

The authors have no financial conflict of interest.

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