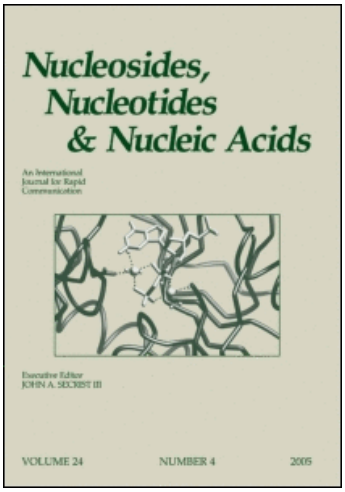

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Modification of Cytokine Milieu by A_{2A} Adenosine Receptor Signaling–Possible Application for Inflammatory Diseases

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ABSTRACT

Pro-inflammatory cytokine TNF-alpha (TNF) production from in vitro lipopolysaccharide (LPS)-stimulated human peripheral blood $CD14^+$ cells (PB-CD14) was inhibited by A_{2A} adenosine receptor (AdoR) ($A_{2A}R$) or $ß_2$ adrenergic receptor (ADR) ($ß_2R$) signaling in a concentration-dependent manner. These inhibitory effects were presumably mediated by the increase in intracellular cAMP. Furthermore $A_{2A}R$ agonist and $ß_2R$ agonist synergistically inhibited the TNF production of LPS-stimulated PB-CD14 cells. These results suggest that the anti-inflammatory effect of extracellular adenosine is, at least in part, due to the modification of the cytokine milieu via A_{2A} signaling, and that the targeting of both $A_{2A}R$ and $ß_2R$ may have strong therapeutic potential for the inflammatory diseases.

Key Words: A_{2A} adenosine purinergic receptor; Beta2 adrenergic receptor; Tumor necrosis factor; Human monocytes; Cyclic AMP; Anti-inflammatories.

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INTRODUCTION

The need to control inflammation is obvious as the prolonged or inappropriate inflammatory responses contribute to the pathogenesis of many diseases including cancer, heart disease, and atherosclerosis. A_{2A} adenosine receptor ($A_{2A}R$) pathway is a good candidate for the regulation of inflammation since the pharmacological activation of $A_{2A}R$ on lymphocytes is reported to stimulate an anti-inflammatory response.[1] Among the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF) may play a central role in human inflammatory diseases such as endotoxin shock and rheumatoid arthritis, which is evidenced by the fact that the blockade of TNF signaling by anti-TNF or anti-TNF receptor antibodies is very effective for the treatment of inflammatory diseases such as rheumatoid arthritis. Because the inhibitory effects of adenosine (Ado) on the secretion of pro-inflammatory cytokines, especially by monocytes and macrophages, have been well documented,[2] we investigated the potential clinical usefulness of Ado together with ß$_2$ ADR (ß$_2$R) stimulation which is widely used in the treatment of inflammatory responses.

MATERIALS AND METHODS

Separation of PB-CD14—After obtaining the informed consent, human peripheral mononuclear cells were isolated by density gradient (Ficoll-Paque Plus; Pharmacia Biotech, Piscataway, NJ) from heparinized peripheral blood obtained from healthy donors. After depletion of $CD4^+$ cells, $CD14^+$ cells were then purified positively using the magnetic beads coated with anti human CD14 (CD14 MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany) and AutoMACS system (Miltenyi Biotec). The purity of the preparation was determined by the flow cytometry and only the preparation with > 95% purity was used for the subsequent assays.

cAMP measurement—PB-CD14 were incubated alone, with selective $A_{2A}R$ agonist CGS21680 (CGS) ± selective $A_{2A}R$ antagonist ZM241385 (ZM; Tocris, Ellisville, MO), or with selective ß$_2$R agonist terbutaline (Terb) ± selective ß$_2$R antagonist ICI 118,551 (ICI) in the presence of non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), and the levels of cAMP were measured using cAMP EIA System (Amersham, Arlington Heights, IL).

Cytokine measurement—PB-CD14 were incubated overnight with 100 ng/ml lipopolysaccharide (LPS; from E. coli 055:B5) to induce TNF production. The cytokine concentration was determined by enzyme-linked immnosorbent assay (Endogen, Woburn, MA). The effects of membrane-permeable cAMP analogue dibutyryl cAMP (dbcAMP), CGS ± ZM, or Terb ± ICI on the LPS-induced TNF accumulation was similarly determined. All the reagents were purchased from Sigma (St. Louise, MO) unless otherwise mentioned.

RESULTS

Functional $A_{2A}R$ and ß$_2$ ADR expression—Since $A_{2A}R$ and ß$_2$R are coupled with Gs protein and are capable of activating the adenylate cyclase which results in the

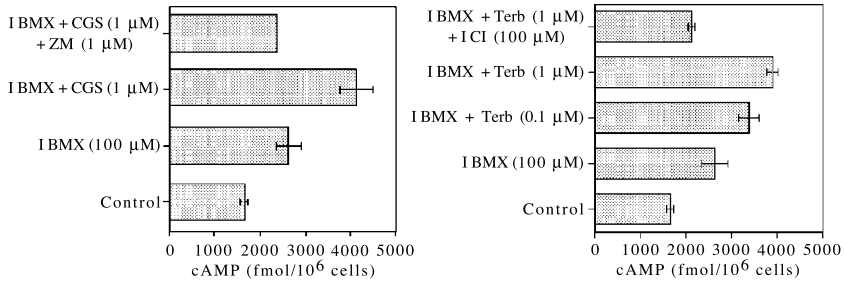

Figure 1. Functional expression of $A_{2A}R$ and $ß_2R$ on PB-CD14. cAMP accumulation of PB-CD14 was observed either by $A_{2A}R$ agonist CGS which was inhibited by $A_{2A}R$ antagonist ZM (right panel), or $ß_2R$ agonist Terb which was inhibited by $ß_2R$ antagonist ICI (left panel).

accumulation of cAMP, the effects of those receptor-mediated signaling were investigated. $A_{2A}R$ and $ß_2R$ mRNA were positive on PB-CD14 by RT-PCR (data not shown). The cells were incubated with CGS ± ZM or Terb ± ICI. On either incubation cAMP was increased, indicating the presence of functional $A_{2A}R$ and $ß_2R$ (Fig. 1).

cAMP effect on TNF-alpha—The effect of dbcAMP, a cell-permeable cAMP analogue, on the TNF production by PB-CD14 cells was tested. The existence of dbcAMP (at 100 μM), either with (dbcAMP → dbcAMP + LPS) or without (dbcAMP + LPS) the preincubation of dbcAMP before the LPS stimulation, completely inhibited the LPS-induced TNF production on these cells, while dbcAMP itself had no effect (Fig. 2).

$A_{2A}R$ and $ß_2R$ signaling on LPS-induced TNF production—The fact that both $A_{2A}R$ and $ß_2R$ were functional on PB-CD14 prompted us to test the effects of $A_{2A}R$ and $ß_2R$ signaling on LPS-induced TNF production. As shown in Fig. 3 (upper panel), $A_{2A}R$ agonist CGS inhibited TNF production in a concentration-dependent manner. The selective $A_{2A}R$ antagonist ZM at 1 μM completely cancelled this inhibitory effect of CGS

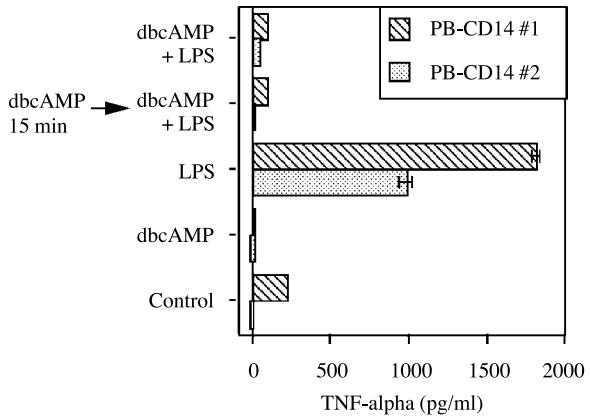

Figure 2. The inhibitory effect of cAMP on the TNF production by PB-CD14.

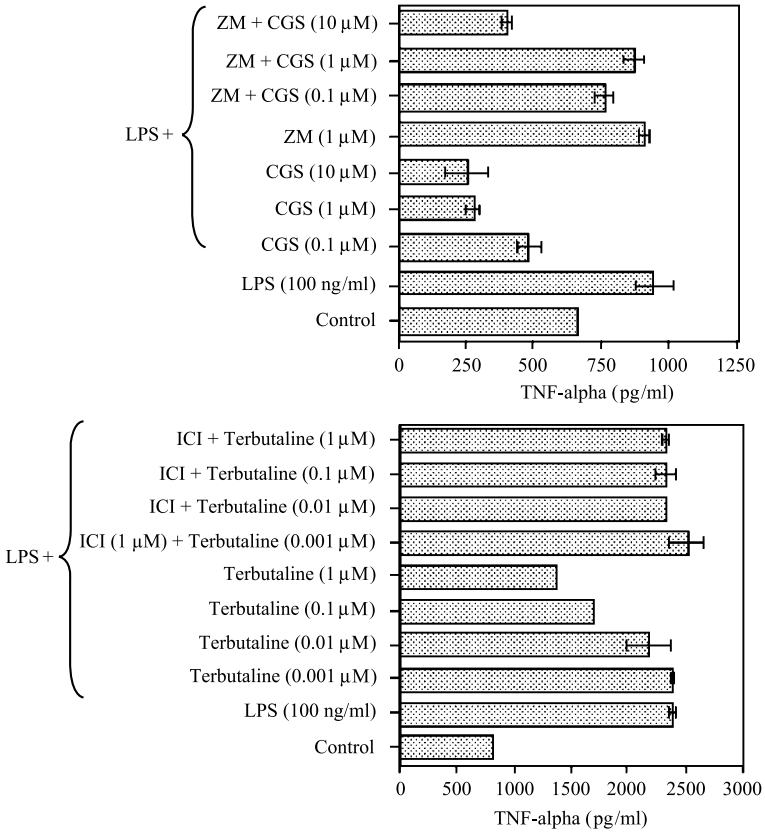

Figure 3. TNF Accumulation was inhibited either by $A_{2A}R$ or $ß_2R$ signaling.

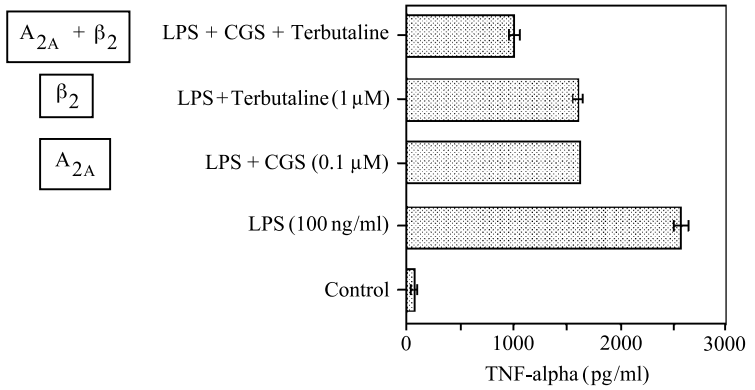

Figure 4. Additive inhibition of TNF production by the simultaneous stimulation of $A_{2A}R$ and $ß_2R$ on human PB-CD14.

at 0.1 and 1 μM and partially blocked even when CGS was more abundant. Similar to $A_{2A}R$ signaling, selective $ß_2R$ agonist Terb inhibited the TNF production of LPS-stimulated PB-CD14 in a concentration-dependent manner, and this effect of Terb was completely cancelled by the selective $ß_2R$ antagonist ICI at 1 μM (Fig. 3, lower panel).

Synergistic inhibition of LPS-induced TNF production by $A_{2A}R$ and $ß_2R$ signaling—Since each of $A_{2A}R$ and $ß_2R$ signaling alone was capable of down-regulating the LPS-induced TNF production, we next investigated if there is a synergistic effect of $A_{2A}R$ and $ß_2R$ to explore the possible therapeutic utilization of the selective $A_{2A}R$ agonist for the treatment of the disease with high TNF-alpha state, such as endotoxin shock, together with the $ß_2R$ agonist such as norepinephrin, which is widely used on the clinical settings. As shown in Fig. 4, each of CGS and Terb at the minimal concentration tested (0.1 and 0.01 μM, respectively) again significantly inhibited the LPS-induced TNF production. As was expected, significantly enhanced inhibition of TNF production was observed when CGS was added together with Terb.

DISCUSSION

$ß_2R$ agonists are clinically widely used for the treatment of the inflammatory disorders such as bronchial asthma. The mechanism of action is mainly explained as follows; $ß_2R$ are coupled with Gs-protein and can increase levels of intracellular cAMP in the smooth muscle cells that results in the bronchial dilatation. The pro-inflammatory cytokine TNF is known to be increased during exacerbation of asthmatic symptoms and directly impairs ß-adrenergic function in airway smooth muscle cells.[3] Since $A_{2A}R$ is also coupled with Gs-protein and can increase levels of cAMP in the cells, Ado via $A_{2A}R$ is a good candidate as an negative regulator of the inflammatory disorders such as asthma directly (i.e. bronchial dilation) and indirectly (i.e. inhibition of TNF). Indeed $A_{2A}R$ signaling has recently been shown to have a non-redundant role in the attenuation of inflammation and tissue damage.[4] When $A_{2A}R$ was genetically inactivated, mutant mice suffered more than wild-type mice from the concanavalin A-induced liver damage and bacterial endotoxin-induced septic shock. In mutant mice secretion of pro-inflammatory cytokines was increased in intensity and duration. Thus we seek the possibility of medical application of $A_{2A}R$ signaling, either alone or together with $ß_2$ agonist, as the negative feedback mechanism for limitation and termination of various inflammatory responses by regulating the production of pro-inflammatory cytokines.

The representative pro-inflammatory cytokine TNF production from LPS-stimulated PB-CD14 was inhibited by $A_{2A}R$ or $ß_2R$ signaling in a concentration-dependent manner (Fig. 3). Because the signaling via $A_{2A}R$ and $ß_2R$ resulted in the increase in the intracellular cAMP (Fig. 1) which was capable of inhibiting the TNF production (Fig. 2), it is concluded that the inhibitory effect of $A_{2A}R$ and $ß_2R$ signaling is mediated by intracellular cAMP. This prompted us to test if the co-stimulation of $A_{2A}R$ and $ß_2R$ could augment their individual ability. As shown in Fig. 4, $A_{2A}R$ agonist and $ß_2R$ agonist had synergistic inhibitory effect on the TNF production of LPS-stimulated PB-CD14.

These results suggest that the anti-inflammatory effect of extracellular Ado is, at least in part, due to the modification of the cytokine milieu via $A_{2A}R$ signaling, and

that the targeting of both $A_{2A}R$ and $ß_2R$ may have strong therapeutic potential for the inflammatory diseases such as asthma and endotoxin shock.

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