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Suppression of inflammation response by a novel A_3 adenosine receptor agonist thio-Cl-IB-MECA through inhibition of Akt and NF-κB signaling

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ABSTRACT

Adenosine, a purine nucleoside, is released from metabolically active cells into extracellular space and plays an important role in various pathophysiological processes. Adenosine regulates many biological responses including inflammation by the interaction with their receptors such as A_1, A_{2A}, A_{2B}, and A_3. Especially, A_3 adenosine receptor (A_3AR) is considered to be expressed in macrophage cells. To the end, A_3AR agonists have been reported to have an anti-inflammatory activity. In our continuous efforts to develop new anti-inflammatory agents, we found a novel adenosine analog, 2-chloro-N^6-(3-iodobenzyl)-4′-thioadenosine-5′-*N*-methyluronamide (thio-Cl-IB-MECA), was a potent human A_3AR agonist. The study was designed to investigate whether thio-Cl-IB-MECA has an anti-inflammatory potential in mouse macrophage RAW 264.7 cells and mouse sepsis model *in vivo*. Thio-Cl-IB-MECA exhibited an effective anti-inflammatory activity. The expression of pro-inflammatory biomarkers including inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), and tumor necrosis factor (TNF-α) was suppressed by the treatment of thio-Cl-IB-MECA in the protein and mRNA levels in lipopolysaccharide (LPS)-stimulated mouse macrophage RAW 264.7 cells. Further examination revealed that thio-Cl-IB-MECA inhibited LPS-induced phosphatidylinositol 3-kinase (PI3 kinase)/Akt activation, NF-kB binding activity, and β-catenin expression. In addition, in *in vivo* LPS-induced mouse endotoxemia model, thio-Cl-IB-MECA exerted the increase of survival rate compared to vehicle-treated mouse. The analysis of the protein levels of iNOS, IL-1β, and TNF-α was also suppressed by the compound-treated groups in lung tissues. These results suggest that thio-Cl-IB-MECA might have an anti-inflammatory activity through the inhibition of pro-inflammatory cytokine expression by modulating PI3K/Akt and NF-κB signaling pathways.

Introduction

Adenosine is produced by the degradation of intracellular ATP and released from the metabolically active cells (Stiles 1990; Linden 2001). Adenosine mediates various physiological functions including cell growth, differentiation, and cell death through binding to selective G-protein coupled membrane surface receptors, designated A_1, A_{2A}, A_{2B}, and A_3. The expressions of these receptor subtypes are dependent on the cell and tissue types, and thus adenosine's effects are pleiotropic. Recent studies suggested that A_3AR is also expressed in macrophage and immune cells and plays a major role in mediating the anti-inflammatory effect of adenosine (Jacobson 1998; Fredholm et al. 2001). Adenosine exhibits the stimulation of phagocytosis of macrophage cells and suppresses the expressions of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 (Hasko et al. 1996; Sajjadi et al. 1996). In addition, adenosine and highly selective A_3AR agonists exerted the anti-inflammatory activity in arthritis animal model through inhibition of NF-κB activation, and thus resulted in the inhibition of pro-inflammatory cytokine production (Baharav et al. 2005; Fishman et al. 2006; Ochaion et al. 2008).

In our ongoing study to develop new A_3AR agonists as promising therapeutic targets, we recently found thio-Cl-IB-MECA as a novel specific A_3AR agonist (Lee et al. 2005; Kim et al. 2008). Previous studies showed that thio-Cl-IB-MECA exhibited the potential antitumor and anti-ischemic activities (Lee et al. 2005; Chung et al. 2006; Kim et al. 2008). In the present study, we designed to investigate the effects of thio-Cl-IB-MECA on the modulation of inflammatory responses mediated by the pro-inflammatory cytokines such as TNF-α, IL-1β, and NO production in

Abbreviations: A_3AR, A_3 adenosine receptor; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; IL-1β, interleukin-1β; IL-6, interleukin-6; NF-κB, nuclear factor-κB; PI3 kinase, phosphatidylinositol 3-kinase; TNF-α, tumor necrosis factor-α.

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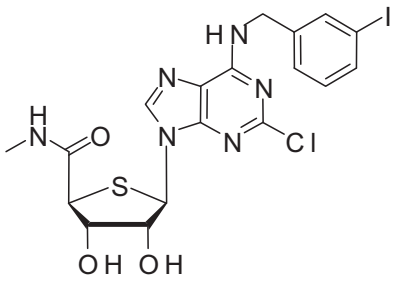

Fig. 1. The chemical structure of thio-Cl-IB-MECA.

lipopolysaccharide-induced murine macrophage cells. In addition, we determined whether the activation of A_3AR with thio-Cl-IB-MECA prevents the lethality evoked by lipopolysaccharide in an endotoxemic mouse model.

Materials and methods

Chemicals

Lipopolysaccharide (LPS, *Escherichia coli* 0111: B4 and *Saccharomyces serratia*), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), antibiotics–antimycotics solution, and mouse monoclonal anti-β-actin antibody were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), non-essential amino acid solution, sodium pyruvate, and L-glutamine were from Invitrogen Co. (Grand Island, NY, USA). Antibodies for IκBα, NF-κB p65, IL-1β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal TNF-α antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Reagents related to PCR were purchased from Promega (Madison, WI, USA). Thio-Cl-IB-MECA (Fig. 1) was synthesized as described by Jeong et al. (2003).

Animals

Five week old male ICR mice were purchased from Central Lab. Animal Inc. (Seoul, Korea). All animal experiments and care were conducted in a manner conforming to the Guidelines of the Animal Care and Use Committee of Ewha Womans University approved by the Korean Association of Laboratory Animal Care (permission number: EWHA2009-2-09).

Cell culture

Mouse macrophage cell line RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Cells were incubated at 37 °C, 5% CO_2 in a humidified atmosphere.

Nitrite assay

To evaluate the inhibitory activity of the test material on LPS-induced NO production, RAW 264.7 cells in 10% FBS-DMEM without phenol red were plated in 24 well plates (5×10^5 cells/mL), and incubated for 24 h. Cells were washed with PBS, replaced with fresh media, and then incubated with 1 μg/mL LPS in a presence or absence of test compounds. After additional 20 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction. Briefly, 180 μL of Griess reagents (0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% H_3PO_4) were added to 100 μL of each supernatant from LPS or sample-treated cells in 96 well plates. The absorbance was measured at 540 nm and nitrite concentration was determined by comparison with a sodium nitrite standard curve. % inhibition was expressed as [1 − (NO level of test samples/NO levels of vehicle-treated control)] × 100. The IC_{50} value, the sample concentration resulting in 50% inhibition of NO production, was determined using non-linear regression analysis (% inhibition versus concentration).

Cytotoxicity assay (MTT assay)

After Griess reaction, MTT solution (final 500 μg/mL) was added to each well and further incubated for 4 h at 37 °C. Media were discarded, and dimethyl sulfoxide (DMSO) was added into each well to dissolve generated formazan. The absorbance was measured at 570 nm and % survival was determined by comparison with control group.

Survival study in LPS-induced sepsis model (endotoxemic mouse model)

Mice (ICR, male, 20–25 g) were injected i.p. with LPS (2 mg/kg, sources from *S. marcescens*) in a volume of 200 μL. Thio-Cl-IB-MECA (0.2 mg/kg or 0.5 mg/kg, each $n = 10$) or vehicle (saline containing 2% DMSO, $n = 10$) were administered orally 30 min before LPS injection. After 20 h later, the second challenge of compounds and LPS (10 mg/kg) was performed. After LPS injection, mice were observed 0.5 h–6 h every hour, and then recorded the survival rate until 7 days.

Tissue sample collection

Mice (ICR, male, 20–25 g) were divided into 3 groups: (1) a normal healthy group (non-treated, $n = 3$), (2) a vehicle treated group (saline containing 2% DMSO), and (3) thio-Cl-IB-MECA (0.5 mg/kg, $n = 3$). Mice were injected i.p. with LPS (2.5 mg/kg) in a volume of 200 μL. Test materials were administered orally 30 min before LPS application. After 8 h LPS injection, lung tissues were collected. Tissue samples were homogenized in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.1% SDS, 50 mM NaF, 50 mM Na_3VO_4, protease inhibitor cocktail in DDW) on ice and centrifuged for 30 min at 12,000 × *g*, and supernatants were collected for analysis. The protein content of tissue lysates was determined by BCA method.

Western blot analysis

RAW 264.7 cells were incubated in the presence or absence of various concentrations of compounds and LPS (1 μg/mL) for the indicated time. To obtain total cell lysates, cells were washed twice with ice-cold PBS and harvested. Cells were lysed in 2× sample loading buffer (250 mM Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 50 mM sodium fluoride, 5 mM sodium orthovanadate, and 2% β-mercaptoethanol). Cell lysates were boiled for additional 5 min at 100 °C and stored at −20 °C. The protein content was determined by BCA method. Equal amounts of cell lysates (40–50 μg) were subjected to 8–12% SDS-PAGE and electro-transferred onto PVDF membranes (Milipore, MA, USA). Membranes were blocked in PBST (PBS with 0.1% Tween-20) containing 5% non-fat dry milk for 1 h at room temperature. After washing three times with PBST, membranes were incubated with primary antibodies against iNOS, β-actin for overnight at 4 °C. Membranes were washed three times with PBST and incubated