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Role of CXCR2 on the immune modulating activity of α-iso-cubebenol a natural compound isolated from the *Schisandra chinensis* fruit

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ABSTRACT

Previously, we demonstrated that α-iso-cubebenol, a natural compound isolated from the fruits of *Schisandra chinensis*, strongly enhances therapeutic efficacy against cecal ligation and puncture challenge-induced sepsis. In this study, we found that α-iso-cubebenol stimulated calcium increase and degranulation in human neutrophils. α-Iso-cubebenol also strongly induced neutrophil chemotaxis, which was completely blocked by a CXCR2 antagonist, SB225002. The increased survival rate by α-iso-cubebenol was also significantly attenuated by SB225002. Taken together, the results indicate that α-iso-cubebenol-induced anti-septic activity was mediated by CXCR2, suggesting CXCR2 as an important target for the regulation of sepsis and inflammation.

1. Introduction

It has been known that sepsis, a systemic inflammatory response, is caused by viable bacteria or bacterial products such as lipopolysaccharide (LPS) [1]. The number of hospitalizations with sepsis in the US has annually increased, reaching around 800,000 in 2007 [2]. Moreover, the overall mortality associated with sepsis ranges from 30% to 70%, and sepsis remains the major cause of death in intensive care units in the US. Currently, no therapeutic agents approved from the US FDA are available, and various studies have been on going to identify efficient target molecules for the development of therapeutic agents against sepsis.

CXCR2 is a well-known chemokine receptor, which is expressed on several leukocytes such as neutrophils, monocytes, and macrophages [3]. CXCL8, an endogenous ligand for CXCR2, binds to CXCR2 in humans [4,5], and mouse chemokines, including CXCL1 (KC, keratinocyte-derived chemokine), bind to mouse CXCR2. Physiologically, the activation of CXCR2 by CXCL8 or CXCL1 induces leukocyte chemotactic migration in neutrophils and monocytes [6,7]. In addition to CXCL8 and CXCL1, N-terminal acetylated tripeptide, Ac-PGP, has been reported to act on CXCR2 [8,9]. Although some reports revealed that neutralizing antibodies against CXCR2 or inhibitory molecules for CXCR2 show therapeutic activity against sepsis, we strongly demonstrated that the activation of CXCR2 by Ac-PGP shows therapeutic efficacy against sepsis [9].

Previously, we isolated a novel natural compound, α-iso-cubebenol, from the fruits of *Schisandra chinensis* and demonstrated that the compound shows therapeutic activity against cecal ligation and puncture (CLP) sepsis model [10,11]. Furthermore, α-iso-cubebenol strongly increased the survival rate in the CLP model by increasing the bactericidal activity of phagocytes, and by attenuating inflammatory cytokine production and blocking leukotycte apoptosis [11]. In this study, we investigated the in vitro activity of α-iso-cubebenol on neutrophil activity. We also examined the role of an important chemokine receptor, CXCR2, on the *in vivo* efficacy of α-iso-cubebenol in a preclinical mouse model of sepsis.

2. Materials and methods

2.1. Materials

α-Iso-cubebenol was purified from the dried fruits of *S. chinensis* as described previously [10]. Peripheral blood mononuclear cell separation medium (Histopaque-1077) was from Sigma (St. Louis,

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MO). Fura-2 pentaacetoxymethylester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR).

2.2. Isolation of human neutrophils

Peripheral blood leukocytes were isolated from healthy donors. Human neutrophils were isolated according to the standard procedures for dextran sedimentation, hypotonic lysis of erythrocytes, and lymphocyte separation medium gradient as described previously [12]. Isolated human neutrophils were used promptly.

2.3. Measurement of intracellular Ca^{2+} increase

The level of $[Ca^{2+}]_i$ was measured using Grynkiewicz's method with fura-2/AM [13,14]. Briefly, prepared cells were incubated with 3 μM fura-2/AM at 37 °C for 50 min in fresh serum-free RPMI 1640 medium under continuous stirring. Following this, 2×10^6 -cells were aliquoted for each assay in Ca^{2+}-free Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 5 mM HEPES, pH 7.3, 10 mM glucose, and 0.2 mM EGTA) and incubated with the indicated concentrations of α-iso-cubebenol. Fluorescence changes at dual excitation wavelengths of 340 nm and 380 nm and at an emission wavelength of 500 nm were measured, and the calibrated fluorescence ratio was translated into $[Ca^{2+}]_i$.

2.4. β-Hexosaminidase secretion assay

The amount of released β-hexosaminidase was measured as described previously [15]. Briefly, neutrophils (2×10^5/well) were cultured overnight in 24-well tissue culture plates. The cells were then washed twice with Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.6 mM glucose, 2.7 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 0.1 g/100 ml BSA, and 25 mM HEPES, pH 7.4) and stimulated with α-iso-cubebenol. The reaction was terminated 20 min after stimulation by placing the plate on ice. The amount of β-hexosaminidase secreted into the medium was determined by incubating 50 μl of supernatant or cell lysate with 25 μl of 5 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide in 0.1 M sodium citrate buffer (pH 3.8) at 37 °C for 2 h. At the end of incubation, 50 μl of 0.4 M Na_2CO_3 was added. Absorbance was monitored at 405 nm. Values are expressed as a percentage of the total β-hexosaminidase present in the cells.

2.5. Chemotaxis assay

Chemotaxis assays were performed using multiwell chambers (Neuroprobe Inc., Gaithersburg, MD) [16]. Briefly, prepared human neutrophils were suspended in RPMI 1640 medium at a concentration of 1×10^6 cells/ml, and 25 μl was placed onto the upper well of the chamber that was separated by a 3 μm polyhydrocarbon filter from an α-iso-cubebenol-containing lower well. After incubation for 90 min at 37 °C, nonmigrated cells were removed by scraping, and cells that migrated across the filter were dehydrated, fixed, and stained with hematoxylin (Sigma, St. Louis, MO). Stained cells were counted per well under light microscope [16].

2.6. Animals and sepsis model

Six week aged male wild type albino institute of cancer research center (ICR) mice were used as an experimental sepsis model as described [17]. For cecal ligation and puncture (CLP), mice were anesthetized with intraperitoneal injections of Zoletil (50 mg/kg) and Rompun (10 mg/kg), after which a small abdominal midline incision was made to expose the cecum. The cecum was then ligated below the ileocecal valve, punctured twice through both surfaces (or once for measurement of cytokine production) using a 22-gauge needle, and then the abdomen was closed. Sham CLP mice were subjected to the same procedure but without ligation and puncture of the cecum. Survival was monitored once daily for 10 days.

2.7. Statistical analyses

Survival data were analyzed using the log-rank test. All other data were evaluated using the ANOVA or *t*-test. The Bonferroni test was used for post hoc comparisons, and statistical significance was set *a priori* at $P < 0.05$.

3. Results

3.1. α-Iso-cubebenol stimulates calcium increase in human neutrophil

Previously, we reported that isolated α-iso-cubebenol strongly protected against mortality induced by CLP [11]. α-Iso-cubebenol also markedly increased superoxide anion production from human neutrophils [11]. We also demonstrated that the production of superoxide anion is mediated by intracellular calcium increase in human neutrophils [18]. To test the possibility that α-iso-cubebenol also stimulates calcium increase in human neutrophils, we treated fura-2/AM-loaded human neutrophils with various concentrations of α-iso-cubebenol. Stimulation of human neutrophils with α-iso-cubebenol induced an increase of intracellular calcium concentration in a concentration-dependent manner, showing maximal activity at 100 μg/ml (Fig. 1A). Intracellular calcium increase can be induced by activation of phospholipase C (PLC) activity or activation of membrane-bound calcium channel [19]. The α-iso-cubebenol-induced calcium increase was almost markedly inhibited by the PLC-selective inhibitor, U-73122, but not by its inactive analogue, U-73343 (Fig. 1B). However, several inhibitors for membrane-bound calcium channels such as SK&F, diltiazem, or nifedifine did not inhibit the α-iso-cubebenol-induced calcium increase (Fig. 1B).

3.2. α-Iso-cubebenol stimulates degranulation in human neutrophils

Activated neutrophil secretes granule contents, which mediate defensive activity in the innate immune response [20]. In this

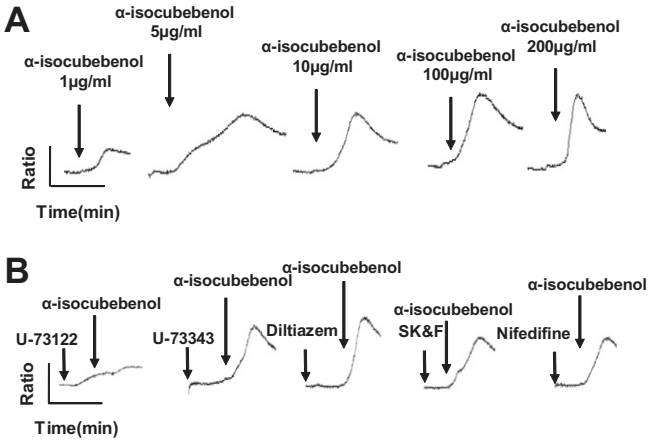

Fig. 1. α-Iso-cubebenol stimulates $[Ca^{2+}]_i$ release in human neutrophils. Human neutrophils were stimulated with various concentrations (1, 5, 10, 50, 100, or 200 μg/ml) of α-iso-cubebenol (A). Human neutrophils were preincubated with U-73122 (5 μM), U-73343 (5 μM), diltiazem (10 μM), SK&F (10 μM), or nifedifine (1 μM), and then stimulated with α-iso-cubebenol (10 μg/ml) (B). The relative $[Ca^{2+}]_i$ are expressed as fluorescence ratios. The data represents three independent experiments (A and B).

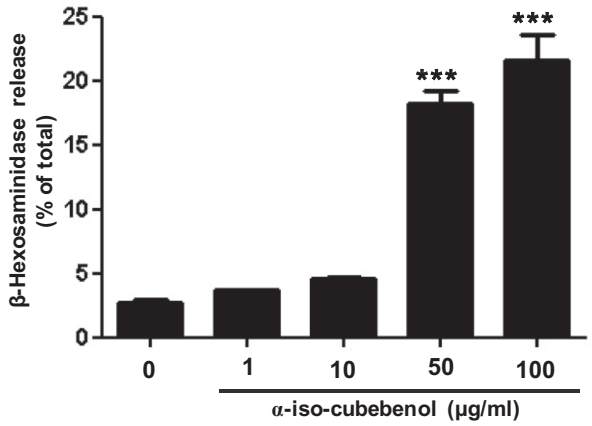

Fig. 2. α-Iso-cubebenol stimulates degranulation activity in human neutrophils. Various concentrations (0, 1, 10, 50, or 100 μg/ml) of α-iso-cubebenol were administered to human neutrophils. The α-iso-cubebenol-induced secretion of β-hexosaminidase was determined. Data are expressed as mean ± SE; $n = 8$. $^{***}P < 0.001$, compared with the value obtained from the untreated control.

study, we investigated the effect of α-iso-cubebenol on degranulation-stimulating activity in human neutrophils. As shown in Fig. 2, stimulation of human neutrophils with various concentrations of α-iso-cubebenol induced degranulation. To test the effect of calcium signaling on α-iso-cubebenol-stimulated degranulation in human neutrophils, we incubated human neutrophils with the calcium chelator, BAPTA/AM, prior to α-iso-cubebenol addition. The α-iso-cubebenol-stimulated degranulation activity was completely inhibited by BAPTA/AM, indicating the involvement of calcium increase in α-iso-cubebenol-stimulated degranulation (data not shown).

3.3. α-Iso-cubebenol stimulates human neutrophil chemotaxis via CXCR2

Neutrophil chemotactic migration is a very essential process in the modulation of innate immunity and inflammatory response [21]. Many chemoattractants regulate neutrophil chemotaxis; pertussis toxin-sensitive G-protein-coupled receptors such as chemokine receptors are activated by these chemoattractants [22,23]. Here we examined whether α-iso-cubebenol would regulate neutrophil chemotaxis. As shown in Fig. 3A, stimulation of human neutrophils strongly induced neutrophil chemotaxis.

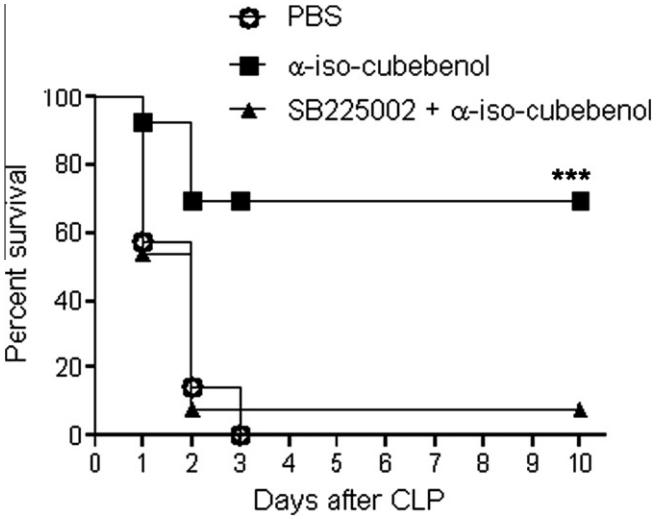

Fig. 4. Role of CXCR2 on α-iso-cubebenol-induced protection against CLP-induced mortality. Vehicle (0.8% DMSO in PBS), α-iso-cubebenol (15 mg/kg), or SB225002 (15 mg/kg) plus α-iso-cubebenol (15 mg/kg) were injected subcutaneously four times into CLP mice at 2, 14, 26, and 38 h post-CLP. Sample size: $n = 9$–10 mice/group. $^{***}P < 0.001$ compared to the vehicle control by ANOVA.

Neutrophils express several chemokine receptors [21]. Among these chemokine receptors, CXCR2, a receptor for CXCL8, has been known to mediate neutrophil chemotaxis and calcium increase [4,6]. Here we tested the possibility of CXCR2 involvement in α-iso-cubebenol-induced neutrophil chemotaxis using a CXCR2-selective antagonist, SB225002. Interestingly, α-iso-cubebenol-induced neutrophil chemotaxis was inhibited by SB225002 (Fig. 3B).

3.4. α-Iso-cubebenol shows anti-septic activity via CXCR2

Since we demonstrated that α-iso-cubebenol has therapeutic effects against sepsis in our previous report [11], and that α-iso-cubebenol-induced neutrophil chemotaxis is blocked by the CXCR2 antagonist (Fig. 3B), we tested the role of CXCR2 on α-iso-cubebenol-induced anti-septic activity. α-Iso-cubebenol-induced increase of survival rate in the CLP sepsis model was completely inhibited by administration of the CXCR2 antagonist (Fig. 4). α-Iso-cubebenol administration-induced attenuation of lung inflammation

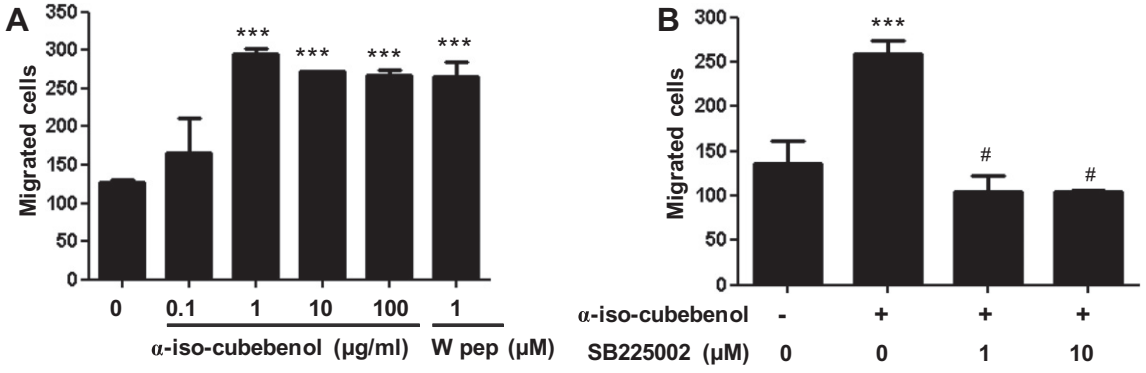

Fig. 3. α-Iso-cubebenol stimulates neutrophil chemotaxis via CXCR2. Human neutrophils (1×10^6 cells/ml of serum-free RPMI) were added to the upper wells of a 96-well chemotaxis chamber, and migration across a 3-μm pore size polycarbonate membrane was assessed after 1.5-h of incubation at 37 °C. Various concentrations (0, 0.1, 1, 10, or 100 μg/ml) of α-iso-cubebenol or 1 μM WKYMVm were used for chemotaxis assay (A). Vehicle or two different concentrations (1 or 10 μM) of SB225002 pretreated cells were subjected to chemotaxis assay with 10 μg/ml of α-iso-cubebenol (B). The number of migrated cells was determined by counting in a high-power field (400×). Data are presented as mean ± SE of three independent experiments performed in duplicate. $^{***}P < 0.001$, compared with the value obtained from the untreated control. $^{\#}P < 0.05$, significantly different from the α-iso-cubebenol alone control (B).

against CLP sepsis was also markedly reversed by administration of the CXCR2 antagonist (data not shown).

4. Discussion

Although mortality rates from sepsis have decreased in recent years, the figure is still around 30% [1,2]. Moreover 1 in 1200 Americans die of severe sepsis annually [1,2]. After Xigris, a therapeutic agent against sepsis approved by the US FDA, has been withdrawn from the market, researchers have been avidly researching for new targets and therapeutic molecules to treat sepsis. In our previous report, we demonstrated that α-iso-cubebenol, a natural compound isolated from the fruits of *S. chinensis*, has strong therapeutic effects against polymicrobial sepsis [11]. In the present study, we demonstrated that the therapeutic effect of α-iso-cubebenol after induction of sepsis by CLP is mediated by an important chemokine receptor, CXCR2. We clearly revealed that the therapeutic effect of α-iso-cubebenol requires compound engagement with CXCR2, since pretreatment of mice with the CXCR2-specific antagonist (SB225002) abolished the efficacy of α-iso-cubebenol in CLP-induced sepsis, thus indicating that α-iso-cubebenol acts via CXCR2 to prevent septic mortality.

Previous studies have indicated that stimulation of CXCR2 by its endogenous ligands such as CXCL8 and CXCL1 induces leukocyte chemotactic migration in neutrophils and monocytes [24,25]. Moreover, clinical data showed that CXCR2 but not CXCR1 is down-regulated by 50% in the neutrophils of sepsis patients compared to normal controls [26]. Since CXCR2 is important for the recruitment of inflammatory cells including neutrophils and monocytes, it has been regarded that blocking of CXCR2 using antibodies or antagonists would show increased survival rate in experimental sepsis models [27]. Novel lipid-conjugated peptide molecules derived from the CXCR2 intracellular region (x1/2pal-3: pal-RTLFKAHMGQKHR), which blocks CXCR2-mediated signaling by CXCL8 or CXCL1, also showed a marked therapeutic effect against cecal ligation and puncture (CLP)-septic mice [28]. Collectively, CXCR2 and its cognate ligands have been considered as important proinflammatory response-inducing targets. However, in our previous report we demonstrated that administration of Ac-PGP, a novel agonist which binds to CXCR2, elicit strong therapeutic efficacy in a CLP sepsis model by increasing bactericidal activity, decreasing inflammatory cytokines, and blocking leukocyte apoptosis [9]. Our previous report and other group's reports collectively suggest that CXCR2 can be differentially regulated in a ligand-selective manner to cause promotion or inhibition of sepsis pathogenesis. In this study, we also exhibited that α-iso-cubebenol shows anti-septic activity via CXCR2. This finding strongly supports our notion that activation of CXCR2 can be a good approach to develop efficient therapeutic agents against sepsis.

In conclusion, α-iso-cubebenol, a novel natural product isolated from the fruits of *S. chinensis*, stimulated neutrophils, resulting in calcium increase, degranulation, and chemotactic migration via CXCR2. Since the α-iso-cubebenol-induced therapeutic effect against sepsis was also blocked by the CXCR2 antagonist, α-iso-cubebenol, and its target, CXCR2, may prove useful in the treatment of polymicrobial sepsis.

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