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Glycyrrhizin derivative inhibits eotaxin 1 production via STAT6 in human lung fibroblasts

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Abstract

We recently demonstrated that glycyrrhizin (GL) and its derivatives down-regulate TNFα- and IL-4-induced eotaxin 1 production by the human fetal lung fibroblast line HFL-1 at protein or mRNA levels. In particular, the GL derivative hetero-30-OH-GL (3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-olean-11,13(18)-dien-30-ol) showed marked inhibition of eotaxin 1 production with less cytotoxicity than 18β-GL. To identify the molecular mechanism of this effect, we focused on the inhibition of the transcriptional factors NF-κB and signal transducer and activator of transcription 6 (STAT6), which regulate eotaxin 1 gene activation. STAT6 phosphorylation and translocation of phospho-STAT6 from cytosol to nuclei were slightly inhibited by 18β-GL and significantly inhibited by hetero-30-OH-GL. While IκBα degradation and translocation of NF-κB p65 to nuclei were not significantly affected by either compound, the stability of eotaxin-1 mRNA was decreased with hetero-30-OH-GL. In addition, eotaxin 1 promoter activity was markedly inhibited by hetero-30-OH-GL. Electrophoretic mobility shift assay (EMSA) confirmed these results. Thus, hetero-30-OH-GL significantly inhibited eotaxin 1 expression by the selective inhibition of IL-4 signal transduction as well as by enhanced mRNA degradation.

Keywords: Glycyrrhizin derivatives; Eotaxin 1; Human lung fibroblast; Signal transducer and activator of transcription 6; Nuclear factor-kappa B

1. Introduction

Eotaxin 1 is a typical eosinophil chemoattractant cytokine belonging to the C–C chemokine family [1]. A large number of studies have indicated that eotaxin 1 plays an important role in Th2-type diseases like asthma and atopic dermatitis. IL-4, one of the Th2-type cytokines, promotes the production of eotaxin 1 and several other chemokines, namely eotaxin 2, eotaxin 3, RANTES, MCP-4, MDC and TARC, by tissue resident non-immune cells such as lung fibroblasts, bronchial epithelial cells and dermal fibroblasts [2–6], and by these means attracts immune cells toward the inflammatory site. Further, stimulation with IL-4 and TNFα in combination synergistically increases the production of eotaxin 1 by activating the transcriptional factors STAT6 and NF-κB [7,8]. A number of studies have reported that glucocorticoids, PPARγ agonists and retinoic acid attenuate eotaxin 1 production [4,6,9–11], but no pathway for this effect has been identified, including any role of STAT6 and NF-κB [10,11].

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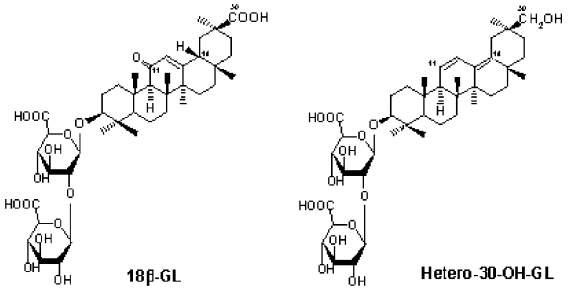
More recently, we have shown that glycyrrhizin (GL) and its derivatives inhibit eotaxin 1 production at protein or mRNA expression levels [12]. GL is an aqueous extract of licorice root which consists of glycyrrhetic acid and two molecules of glucuronic acid at the C-3 position (Fig. 1). A GL preparation, Stronger Neo-Minophagen C (SNMC), has long been used for the treatment of chronic hepatitis in Japan [13], and the activity of GL and its derivatives in their targeting of liver [14–16] and other diseases [12,17,18] has been widely investigated.

In the present study, we investigated the mechanism of action of 18β-GL and (3β-[(2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranuronosyl)oxy]-olean-11,13(18)-dien-30-ol) (hetero-30-OH-GL), a GL derivative considered to have therapeutic potential, on the inhibition of TNFα-and IL-4-induced eotaxin 1 production mediated by the activation of transcriptional factors NF-κB and STAT6.



Eotaxin 1 production (ng/mL) by TNFα and IL-4 stimuli for 48 hr

Concentration (μg/mL)	18β-GL	Hetero-30-OH-GL
0	12	12
1	11±3.4	8.1±0.6**
10	10.1±0.9	7.9±1.3*
100	9.3±0.7	0.6±0.8**
1000	7.2±0.7**	
IC_{50}	>1000	33
CC_{30}	>1000	>100

* P<0.05 **P<0.01

Fig. 1. Structure of 18β-GL and Hetero-30-OH-GL and their effects on TNFα plus IL-4-induced eotaxin 1 production. Eotaxin 1 levels released during stimulation of HFL-1 cells (5×10^4 cells) with TNFα (10 ng/mL) plus IL-4 (20 U/mL) and various concentrations of each GL compound for 48 h at 37 °C were calculated from ELISA data ($n=3$) reported previously [12]. Statistically different from the effect of TNFα plus IL-4 at * $P<0.05$, ** $P<0.01$ level according to Student's *t*-test. Inhibitory concentration (IC_{50}) against eotaxin 1 production of each GL compound and their cytotoxicity concentration (CC_{30}) values assessed by LDH assay were also excerpted from the

2. Materials and methods

2.1. Cell culture and materials

Human fetal lung fibroblasts (HFL-1, from normal embryonic lung) were purchased from Riken Cell Bank (Saitama, Japan). The cells were cultured in Ham' F-12 (Gibco, Grand Island, NY) supplemented with 15% heat-inactivated fetal bovine serum, penicillin G 100 units/mL, and streptomycin 100 μg/mL in a humidified 5% CO_2/95% air atmosphere at 37 °C. Human recombinant TNFα was kindly provided by Dainippon Pharmaceutical Co. (Suitashi, Osaka, Japan). Human recombinant IL-4 was purchased from Peprotech EC (London, UK). 18β-GL and hetero-30-OH-GL were prepared as described previously [12].

2.2. Western blot analysis

HFL-1 cells (approximately 3×10^6) were treated with TNFα (10 ng/mL) plus IL-4 (20 U/mL) and each GL compound for 0–60 min and washed with PBS. Whole cell lysates and cytoplasmic and nuclear extracts were prepared as described elsewhere [19–21]. Sample protein concentrations were determined by Bradford assay, and the samples were stored at −80 °C. Whole cell lysate (20 μg), cytoplasmic extract (20 μg) or nuclear extract (10 μg) were boiled for 5 min in an equal volume of 2× Laemmli's sample buffer, then separated by electrophoresis on 10–12% SDS-PAGE and transferred to a nitrocellulose transfer membrane (Protoran, Schleicher and Schuell BioScience, Germany). Membranes were blocked in 5% nonfat milk powder in TPBS (0.05% Tween 20 containing PBS) at 4 °C overnight, then incubated with the following antibodies diluted in TPBS for 1 h at room temperature with continuous shaking: rabbit polyclonal anti-phospho-STAT6 Ab (Tyr641) (1:1000, Cell Signaling); rabbit polyclonal anti-STAT6 Ab (1:500, SC-621, Santa Cruz); rabbit polyclonal anti-NF-κB p65 Ab (1:1000, SC-372, Santa Cruz); rabbit polyclonal anti-IκBα Ab (1:500, SC-371, Santa Cruz); or goat polyclonal anti-actin Ab (1:500, SC-1615, Santa Cruz). After washing with TPBS (5 times × 5 min each; room temperature), membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-goat immunoglobulin (DAKO) diluted 1:1000 in TPBS containing 1% nonfat milk powder for 1 h at room temperature with continuous shaking. After washing with TPBS (5 times × 5 min each; room temperature), immunoreactive bands were visualized with an ECL detection system (Amersham).

2.3. Plasmids, transient transfection and luciferase assay

A 442-bp eotaxin 1 promoter fragment (site −406 to +36) was amplified by PCR using a sense primer (5′-GGTAC-CATGTGAACACAGGAATC-3′) containing a restriction site for *Kpn*I and an antisense primer (5′-AAGCTTTGGCGT-GAGAGGTGG-3′) containing a restriction site for *Hin*dIII