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Suppressive Effects of Triterpenoids on CINC-1 Induction in Interleukin-1β-Stimulated Rat Fibroblast NRK-49F Cells

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CINC-1 is a member of chemokine family with chemotactic and activating properties to neutrophils. CINC-1 induction in IL-1β-stimulated rat fibroblast NRK-49F cells was quantitated by a sensitive ELISA. CINC-1 production was increased up to 135 ng/ml from basal 2-6 ng/ml by stimulation with IL-1β. Steroidal anti-inflammatory drugs including dexamethasone and prednisolone exhibited potent suppressive effects on IL-1β-induced CINC-1 production. Among 39 kinds of natural triterpenoids tested, acacigenin B exhibited the highest suppressive effects with about 10 μM to be 50% of inhibition on the CINC-1 induction. The suppressive potency of acacigenin B on IL-1β-induced CINC-1 production was about 10-fold less than that of the steroidal anti-inflammatory drugs.

Key words : CINC-1 induction, IL-1β-stimulated NRK-49F cells, Triterpenoids, Anti-inflammatory drugs, ELISA

INTRODUCTION

Migration of leukocytes from the circulation to accumulate at inflamed sites and activation of the cells are central events of acute inflammatory reactions. A variety of mediators known as chemoattractants have been identified which can result in accumulation and activation of leukocytes at sites of inflammation (Cramer, 1992). Migratory responses of leukocytes are induced by chemoattractant doses that are much lower than those required to activate the cells. Lipid metabolites PAF and leukotriene B4, peptide fMLP, complement fragment C5a, and chemokines are well known as chemoattractants (Snyderman and Uhing, 1992).

Chemokines, chemotactic cytokines, have structural similarity on the conservation of four cysteines in all proteins, and are divided into C-X-C and C-C subfamilies depending on the arrangement of two N-terminal cysteines (Schall, 1994). Human C-X-C subfamily includes interleukin (IL)-8, neutrophil-activating proteins, platelet-derived factors, stromal cell-derived factor 1 (SDF-1), and GRO proteins, and C-C subfamily includes monocyte-chemotactic proteins, RANTES, and macrophage inflammatory proteins (MIP). The C-X-C chemokines are known to preferentially attract neutophils, but C-C chemokines induce the migration of monocytes but not neutrophils (Baggiolini *et al.*, 1992). In addition to important roles in inflammatory diseases, chemokine receptors ware recently indentified as coreceptors for HIV-1 infection (D'Souza and Harden, 1996). C-X-C chemokine receptor-4 (CXCR-4) responding to SDF-1 is a second receptor for M-tropic HIV-1, and C-C chemokine receptor-5 (CCR-5) responding to MIP-1α, MIP-β, and RANTES is a second receptor for T-tropic HIV-1 (Dragic *et al.*, 1996; Feng *et al.*, 1996). Thereby, chemokines inhibit HIV-1 infection by interfering with the virus-cell fusion reaction, subsequent to CD4 binding (Cocchi *et al.*, 1995).

Cytokine-induced neutrophil chemoattractants of CINC-1, CINC-2α, CINC-2β, and CINC-3 are rat chemokines belong to C-X-C subfamily (Nakagawa *et al.*, 1994). CINC-1 was originally indentified in culture supernatant of IL-1β-stimulated rat kidney epithelioid NRK-52E cells (Watanabe *et al.*, 1989). Rat chemokines of CINC-2α, CINC-2β, and CINC-3 were identified by analysis of cDNA obtained from total RNA of LPS-stimulated rat macrophages (Nakagawa *et al.*, 1994). All members of CINC family exhibit high degree of sequence homology to human GRO proteins and mouse MIP-2 rather than to human IL-8. The CINC family have strong chemotactic and activating properties of rat and human neutrophils (Watanabe *et al.*, 1991). Rat neutrophils are known to have two receptors mediating chamotactic activity of CINC family, one responding to all of CINC family and the oth-

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er specific to CINC-3 (Shibata *et al.*, 1995).

Only dexamethasone is known to have suppressive effect on chemokine IL-8 induction (Mukaida *et al.*, 1992). As a part of our screening study to identify anti-inflammatory agents from natural products, triterpenoids which have steroidal structures similar to dexamethasone were selected as samples, and their suppressive effects on CINC-1 induction in IL-1β-stimulated rat fibroblast NRK-49F cells were analyzed in this study. Among 39 kinds of triterpenoids tested, a-cacigenin B exhibited the highest suppressive effect with about 10 μM to be 50% of inhibition on the CINC-1 induction.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, and bovine serum albumin were purchased from Sigma Chemical Co., and penicillin G potassium, streptomycin sulfate and *o*-phenylenediamine from Wako Chemical Ind. Ltd. Fetal bovine serum was obtained from HyClone Lab., and Streptavin-horseradish peroxidase from Caltag.

Culture of NRK-49F cells

Rat fibroblast NRK-49F cells were grown in DMEM (13.4 mg/ml Dulbecco's modified Eagle's medium, 24 mM $NaHCO_3$, 10 mM HEPES, 143 units/ml penicillin G potassium, 100 μg/ml streptomycin sulfate, pH 7.1) containing 5% fetal bovine serum (FBS) with 5% CO_2 at 37°C. When the fibroblasts were confluent, PBS buffer (0.2 M NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.8 mM K_2HPO_4) containing 0.25% trypsin and 0.02% EDTA was added to detach the cells from a culture dish (Falcon). The detached cell suspension was about 10-fold diluted with DMEM containing 5% FBS, and then subjected to centrifugation at 250×g for 10 min at 4°C. After washing with DMEM once, NRK-49F cells were resuspended in 5 ml of DMEM containing 5% FBS, stained with trypan blue, and counted their numbers by using a microscope. Viability of the NRK-49F cells was more than 99% in all preparations. The NRK-49F cells were diluted to 5×10^4 cells/ml with DMEM containing 5% FBS.

IL-1β stimulation and sample treatment

The diluted NRK-49F cells were dispensed to 1×10^4 cells per well of a 96-well culture plate (Falcon), and then incubated at 37°C with 5% CO_2 for 48 hrs. The NRK-49F cells were washed once with DMEM containing 0.1% BSA, treated with 100 μl of 2×10^{-10} M IL-1β and 100 μl of designated concentration of each sample, and then incubated at 37°C with 5% CO_2 for 24 hrs. Control group was treated with IL-1β only, and blank group with DMEM containing 0.1% BSA instead of IL-1β and samples. The samples were dissolved in 100% dimethyl sulfoxide, and then serially diluted with DMEM containing 0.1% BSA to have less than 0.1% dimethyl sulfoxide when designated concentrations of samples were treated.

ELISA of CINC-1

NRK-49F cells in the 96-well culture plate were centifuged at 500×g for 30 min at 4°C. To analyze CINC-1 amount in the supernatants, a sandwich ELISA was carried out as follows. A 96-well assay plate (Falcon) was coated with 80 μl per well of 5 μg/ml polyclonal rabbit anti-CINC-1 in 50 mM carbonate buffer (pH 9.6), and then incubated at 37°C for 3 hrs. The assay plate was washed three times with PBS buffer containing 0.05% Tween-20, added with 200 μl of 1% BSA per well, and incubated at 4°C for overnight. After washing three times with PBS buffer containing 0.05% Tween-20, the assay plate was added with 80 μl of the culture supernatant per well, and then incubated at 37°C for 1 hr. The assay plate was washed three times with PBS buffer containing 0.05% Tween-20, added with 80 μl per well of 1 mg/ml biotinylated anti-CINC-1 IgG in PBS containing 0.5% BSA, and then incubated at 37°C for 1 hr. After washing three times with PBS buffer containing 0.05% Tween-20, the assay plate was added with 80 μl per well of 10,000-fold diluted streptavidin-horseradish peroxidase in PBS buffer containing 0.05% Tween-20. After incubation at 37°C for 30 min, the assay plate was extensively washed with PBS buffer containing 0.05% Tween-20. One hundred μl of a substrate solution (1% *o*-phenylenediamine, 0.02% hydrogen peroxide, 50 mM sodium citrate, 100 mM phosphate buffer, pH 5) was added to each well of the assay plate. After incubation at room temperature for 10 to 30 min, the assay plate was added with 100 μl per well of 4 N H_2SO_4, and then subjected to measurement of absorbance at 492 nm (A_{492}) by using a microplate reader. Inhibitory effect of each sample on CINC-1 induction was expressed as % of inhibition, [1-(sample A_{492}/control A_{492})] ×100.

RESULTS AND DISCUSSION

CINC-1 induction in IL-1β-stimulated NRK-49F cells

The CINC-1, a member of chemokines, is known to be induced in LPS, IL-1 or TNF-stimulated rat kidney epithelioid NRK-52E cells, TNF-stimulated rat fibroblast NRK-49F cells, and LPS-stimulated rat peritoneal macrophages (Watanabe *et al.*, 1990; Nakagawa *et al.*, 1993; Lee *et al.*, 1995). The NRK-49F cells were stimulated with different concentrations of IL-1β, and then

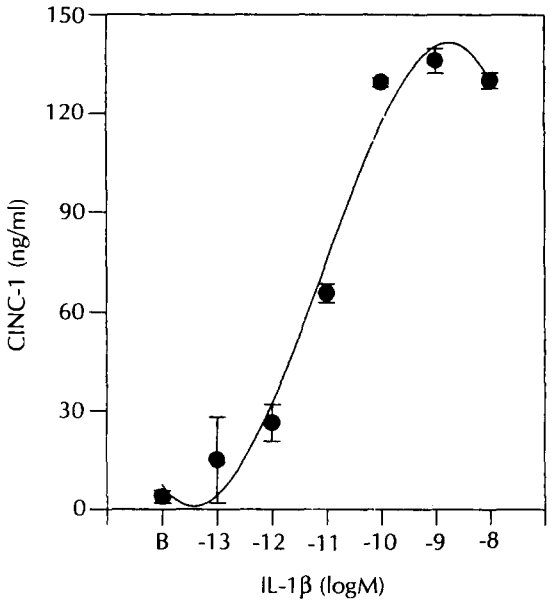

Fig. 1. The CINC-1 induction in IL-1β-stimulated NRK-49F cells. The cells were treated with 1×10^{-13} to 1×10^{-8} M of IL-1β. Lane B indicates basal level of CINC-1 in NRK-49F, and IL-1β (logM) means a logarism of molar concentration of IL-1β. Amounts of CINC-1 in the cuture supernatants were estimated by a sandwich ELISA.

amounts of CINC-1 in culture supernatants were quantitated by a sensitive ELISA (Fig. 1). NRK-49F cells contained 2 ng/ml to 6 ng/ml of CINC-1 as a basal level. When NRK-49F cells were stimulated with 1×10^{-13} M of IL-1β, CINC-1 was not significantly induced. However, NRK-49F cells stimulated with 1×10^{-12} M of IL-1β produced about 26 ng/ml of CINC-1, more 5-fold amount than the basal level. CINC-1 production in NRK-49F cells was dose-dependent of IL-1β treated. Maximal CINC-1 induction with 135 ng/ml was obtained when NRK-49F cells were stimulated with 1×10^{-9} M of IL-1β. NRK-49F cells stimulated with 1×10^{-13} M to 1×10^{-8} M of IL-1β did not exhibit any significant change in their viability and morphology. Thus, IL-1β with 1×10^{-10} M as a final concentration was treated to NRK-49F cells to analyze suppressive effects on CINC-1 induction by anti-inflammatory drugs and triterpenoids.

Suppressive effects by anti-inflammatory drugs

As shown in Table I, both steroidal and nonsteroidal anti-inflammatory drugs exhibited significant suppressive effects on CINC-1 induction in IL-1β-stimulated NRK-49F cells. Steroidal anti-inflammatory drugs of betamethasone, dexamethasone, and triamcinolon at 1 μM exhibited about 50% of inhibition compared with the control, and prednisolone at the same concentration did 66% of inhibition on IL-1β-induced CINC-1 production. All of the steroidal anti-inflammatory drugs at 1 μM as a final concentration did not change the viability and morphology of IL-1β-stimulated NRK-49F cells. The steroidal drugs are known to implicate in gene regulation of certain proteins through interaction with glucocorticoid-responding DNA sequence as their

Table I. Inhibition on CINC induction

Compound	% of Inhibition[1)]
Anti-inflammatory drugs	
Aspirin	21±3
Betamethasone	52±3
Dexamethasone	51±4
Diclofenac	18±3
Indomethacin	8±1
Piroxicam	17±4
Prednisolone	66±6
Sulindac	14±4
Triamcinolon	54±2
Triterpenoids from plants	
Acacigenin B	37±1
Acetylaleuritolic acid	<0
Aleuritolic acid	18±1
β-Amyrin	23±2
β-Amyrin acetate	17±2
Araloside A	<0
Azukisaponin V	<0
Betulic acid	15±4
Dioscin	<0
Diosgenin	<0
Erythrodiol	<0
Glycyrrhetic acid	<0
Gracillin	15±2
α-Hederin	<0
Jaligonic acid	<0
Methyl protogracillin	10±7
Myricadiol 3-acetate	<0
Nigaichigoside F1	20±2
Oleanolic acid	<0
Oleanolic acid glucopyranosyl ester	16±2
Oleanolic acid 3-*O*-arabinose	17±3
Oleanolic acid acetate	<0
Oleanolic acid methyl ester	<0
β-Peltoboykinolic acid	17±1
Phytolaccagenic acid	17±1
Phytolaccagenin	<0
Phytolaccoside B	<0
Phytolaccoside E	<0
Phytolaccoside F	<0
Phytolaccoside I	<0
Pulsatilla saponin A	<0
Pulsatilla saponin F	<0
Pulsatilla saponin H	<0
(25*S*)-Ruscogenin	<0
Stipuleanoside R1	24±1
Stipuleanoside R2	20±1
Suavissimoside R1	<0
Trillin	22±4
Ursolic acid	<0

[1)]Data are represented as mean±standard error (n=6) where each sample treated was 1 μM as the final concentration.

anti-inflammatory and immunoregulatory mechanisms. As the regulatory effects on cytokine network, steroidal drugs inhibit the production of a variety of cytokines including IL-1, IL-2, IL-3, IL-6, IL-8, TNF, and interferon-γ, but increase numbers of IL-1 and IL-6 receptors (Goldstein *et al.*, 1992). Dexamethasone is known to inhibit the IL-1-induced IL-8 production in human fibrosarcoma at transcription level (Mukaida *et al.*, 1992). Therefore, suppressive effects of steroidal anti-inflammatory drugs on CINC-1 induction in IL-1β-stimulated NRK-49F cells in this study would be attributed to downregulation of CINC-1 gene. Nonsteroidal anti-inflammatory drugs of aspirin and indomethacin did not inhibit the CINC-1 induction in LPS-stimulated peritoneal macrophages (Lee *et al.*, 1995; Min *et al.*, 1996). However, nonsteroidal drugs of aspirin, dichlofenac, indomethacin, piroxicam, and sulindac at 1 μM exhibited less than 20% of inhibition on CINC-1 induction in IL-1β-stimulated NRK-49F cells in this study.

Suppressive effects by triterpenoids

Inhibitory effects of triterpenoids on CINC-1 induction in IL-1β-stimulated NRK-49F cells were estimated (Table I). Among 39 kinds of triterpenoids, 14 samples exhibited significant suppression on IL-1β-induced CINC-1 production. Acacigenin B exhibited the highest suppressive effects on the CINC-1 induction. At 1 μM of final concentration, β-amyrin, nigaichigoside F1, stipuleanoside R1, stipuleanoside R2, and trillin exhibited about 20% to 24% of inhibition on the CINC-1 induction, and aleuritolic acid, β-amyrin acetate, betulic acid, gracillin, oleanolic acid glucopyranosyl ester, oleanolic acid 3-*O*-arabinose, β-peltoboykinolic acid, and phytolaccagenic acid did less than 20% of inhibition compared with the control. Acetylaleuritolic acid, araloside A, azukisaponin V, dioscin, diosgenin, erythrodiol, glycyrrhetic acid, α-hederin, jaligonic acid, myricadiol 3-acetate, oleanolic acid, oleanolic acid acetate, oleanolic acid methyl ester, phytolaccagenin, phytolaccosides B, E, F, and I, pulsatilla saponins A, F, and H, ruscogenin, suavissimoside R1, and ursolic acid at 1 μM did not inhibit the CINC-1 induction at all. Unfortunately, we could not correlate the structure-activity relationship of triterpenoids on CINC-1 induction.

Suppression on CINC-1 induction by acacigenin B

Acacigenin B and nigaichigoside F1 did exhibit the similar level of suppressive effects on CINC-1 induction in LPS-stimulated rat peritoneal macrophages (Min *et al.*, 1996). However, acacigenin B was much more potent than nigaichigoside F1 in suppressive effect on IL-1β-induced CINC-1 production of rat fibroblast NRK-49F cells. Acacigenin B used in this study was isolated from *Albizzia julibrissin*, a herbal medicine

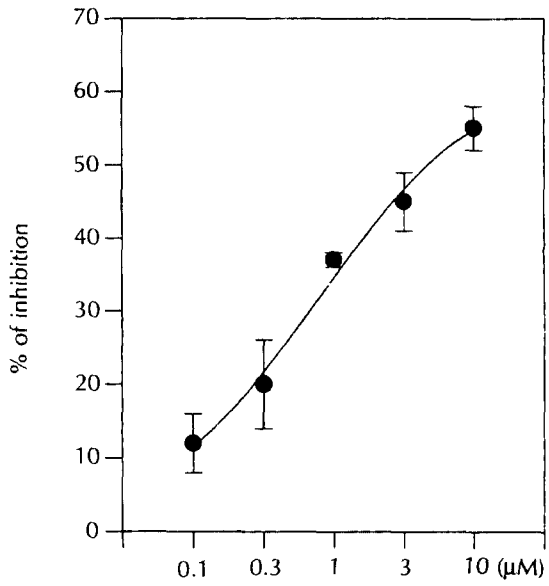

Fig. 2. Dose-dependent inhibition of acacigenin B on IL-1β-induced CINC-1 production.

(Kang and Woo, 1981). Acacigenin B exhibited dose-dependent inhibition on CINC-1 induction in IL-1β-stimulated NRK-49F cells (Fig. 2). Inhibitory effect on the CINC-1 induction by acacigenin B was 12% of inhibition at 0.1 μM, 37% of inhibition at 1 μM, and 55% of inhibition at 10 μM. Acacigenin B did not exhibit any significant change on the viability and morphology of NRK-49F cells stimulated with IL-1β. Therefore, acacigenin B is about 10-fold less potent than steroidal anti-inflammatory drugs in the suppressive effect on IL-1β-induced CINC-1 production of NRK-49 cells. Possible mechanism of the suppressive effect by acacigenin B could be speculated as transcriptional downregulation of CINC-1 gene, IL-1β receptor antagonism, or interference of IL-1β and its receptor complex-mediated signal transduction, which will be elucidated in a future study.

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