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In vitro anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone, from *Saussurea lappa*

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Abstract

We investigated in vitro anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone from *Saussurea lappa*, on tumor necrosis factor (TNF)-α and nitric oxide (NO) release, and lymphocyte proliferation. Cynaropicrin strongly inhibited TNF-α release from lipopolysaccharide-stimulated murine macrophage, RAW264.7 cells, and differentiated human macrophage, U937 cells, proved to produce notable amount of TNF-α. It also potently attenuated the accumulation of NO released from lipopolysaccharide- and interferon-γ-stimulated RAW264.7 cells in a dose-dependent manner. In addition, the immunosuppressive effects of the compound on lymphocyte proliferation in response to mitogenic stimuli were examined. Cynaropicrin also dose-dependently suppressed the proliferation of lymphocytes from splenocytes and interleukin-2-sensitive cytotoxic T lymphocyte, CTLL-2 cells, stimulated by lipopolysaccharide, concanavalin A, phytohemagglutinin and interleukin-2. However, treatment with sulphydryl compound, L-cysteine, abrogated all these inhibitory effects. These results suggest that cynaropicrin may participate in the inflammatory response by inhibiting the production of inflammatory mediators and the proliferation of lymphocytes and its inhibitory effect is mediated through conjugation with sulphydryl groups of target protein(s).

Keywords: Cynaropicrin; Anti-inflammatory effect; TNF-α (tumor necrosis factor-α); Nitric oxide (NO) release; Lymphocyte proliferation; Immunosuppressive effect

1. Introduction

Sesquiterpene lactones constitute a large and diverse group of plant constituents. These components are known for the main materials having various biological activities, including anti-tumor, anti-ulcer, anti-inflammatory, neurocytotoxic and cardiotonic activities (Robles et al., 1995). For examples, costunolide and dehydrocostus lactone exhibited inhibitory effects on killing function of cytotoxic T lymphocytes (Taniguchi et al., 1995), nitric oxide (NO) production (Park et al., 1996), tumor necrosis factor (TNF)-α production (Lee et al., 1999), and hepatitis B virus surface antigen expression (Chen et al., 1995). Helenalin alleviated carrageen-induced edema of rat hindfeet (Hall et al., 1979) and suppressed cancer cell growth (Hall et al., 1977). Parthenolide and encelin showed strong inhibitory effects on the expression of cyclooxygenase as well as TNF-α (Hwang et al., 1996). The inhibitory effect of these sesquiterpene lactones was known to be due to a α-methylene γ-butyrolactone group, which is chemically reactive (Hwang et al., 1996; Bork et al., 1997; Hehner et al., 1998). In recent our studies, from *Saussurea lappa*, a well-known traditional herbal medicine in Korea and China, the active principles on TNF-α production and cytokine-induced neutrophil chemoattractant-1 were also elucidated as the sesquiterpene lactones such as cynaropicrin, reynosin and santamarine with α-methylene γ-butyrolactone group (Cho et al., 1998b; Jung et al., 1998). Among them, cynaropicrin displayed much higher inhibitory potency on TNF-α release from murine macrophages compared to the other compounds.

Macrophages and lymphocytes play an important role in host acute or chronic inflammation (Panayi et al., 1992; Correll et al., 1997). Under these conditions, they are

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proliferated and activated by inflammatory signals such as some bacterial products including lipopolysaccharide, phytohemagglutinin and concanavalin A (Weinstein et al., 1992; Geng et al., 1995). As a result, they secret a number of pro-inflammatory mediators such as cytokines (TNF-α, interleukin-1 and-6) and eicosanoids (prostaglandin E_2 and leukotriene B_4) as well as reactive oxygen and nitrogen intermediates including NO (Ding et al., 1988; Lee et al., 1994).

In view of these, we investigated the inhibitory effect of cynaropicrin on the production of major pathophysiological mediators such as TNF-α and NO, and on the proliferation of lymphocytes (CD4 + and CD8 +), to demonstrate in vitro anti-inflammatory effects of the compound. In addition, since it was reported that α-methylene-γ-butyrolactone moiety by which inhibitory activities were mediated (Hwang et al., 1996; Hehner et al., 1998) is a very important functional group for irreversible binding to the sulphydryl groups of target protein(s), we also evaluated the significance of the moiety in in vitro pharmacology of cynaropicrin using sulphydryl compound, L-cysteine.

2. Materials and methods

2.1. Animals

Eight-week-old BALB/c male mice were purchased from B&K Universal (Fremont, CA, USA). The BALB/c mice were maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied ad libitum.

2.2. Materials

Cynaropicrin (Fig. 1, molecular weight: 346.38) was purified from root of *S. lappa* as described by Cho et al. (1998b). A77,1726, the bioactive metabolite of leflunomide, was supplied from Department of Chemistry in Daewoong R&D center (Sungnam, Korea). Prednisolone, dibutyryl cyclic AMP (dbcAMP), L-cycloserine, L-cysteine, sulfanilamide, *N*-[1-naphthyl]-ethylenediamine dihydrochloride, nitro-L-arginine methyl ester (L-NAME), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), recombinant human interferon-γ, recombinant human interleukin-2, concanavalin A, phytohemagglutinin, lipopolysaccharide (*Escherichia coli* 0111:B4) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum, penicillin, streptomycin and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 cells, a murine macrophage cell line, U937 cells, a human promonocytic cell line, and CTLL-2 cells, mouse cytotoxic interleukin-2-dependent T cell line, were purchased from American Type Culture Collection (Rockville, MD, USA). All other chemicals were of reagent grade.

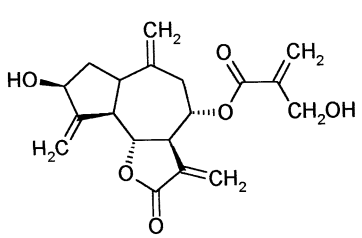

Fig. 1. The chemical structure of cynaropicrin isolated from *S. lappa*.

2.3. Cell culture

RAW264.7 and U937 cells were maintained in RPMI1640 supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 10% fetal bovine serum. In case of CTLL-2 cell maintenance, the cells were cultured with the same medium containing 25 U/ml of interleukin-2. Cells were grown at 37°C and 5% CO_2 in humidified air.

2.4. TNF-α release in vitro

2.4.1. Stimulation of RAW264.7 cells

The inhibitory effect of cynaropicrin on TNF-α release was determined as previously descried (Cho et al., 1998b). Cynaropicrin was solubilized with vehicle (89.9% propylene glycol, 10% ethanol and 0.1% dimethyl sulfoxide) were diluted with RPMI1640. The final concentration of vehicle was never exceeded 0.05% in the culture medium. In these conditions, none of the solubilization solvents altered TNF-α production in RAW264.7 cells. Before stimulation with lipopolysaccharide (1 μg/ml) and testing samples, RAW264.7 cells (2×10^6 cells/ml) were incubated for 18 h in 24 well plates under the same conditions. Stimuli and the various concentrations of testing samples were then added to the wells for 6 h. Supernatants were then collected and assayed for TNF-α content using mouse TNF-α enzyme-liked immunosorbent assay (ELISA) kit (Amersham, Little Chalfont, Buckinghamshire, UK).

2.4.2. Differentiation and stimulation of U937 cells

Differentiation and stimulation of U937 cells were performed with the previous method (Sajjadi et al., 1996). Human U937 cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum. To differentiate U937 cells, 2×10^6 cells/ml were treated with PMA of 20 ng/ml for 24 h. The PMA was removed by washing and adherent cells were then allowed to recuperate for 40 h. The recuperated cells were subsequently incubated with lipopolysaccharide of 1 μg/ml for 6 h with cynaropicrin and positive control drugs. Supernatants were harvested and assayed by ELISA kit for human TNF-α from Amersham Life Science.

2.5. Determination of NO release

RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^6 cells/ml in RPMI1640 supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 10% fetal bovine serum. After preincubation for 18 h, the various concentrations of cynaropicrin or positive control drug with lipopolysaccharide (1 μg/ml) were incubated for 24 h under the same conditions (Ding et al., 1988).

2.6. Measurement of nitrite concentration

Nitrite in culture supernatants were measured, as previously described by Ding et al. (1988), by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenediamine in 5% phosphoric acid) to 100-μl samples of medium, respectively, for 10 min at room temperature. The OD at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The nitrite concentration (μM) was calculated from sodium nitrite standard curve. The detection limit of the assay is 0.5 μM.

2.7. Splenocyte preparation

Splenocytes were prepared from the spleens of mice killed by cervical dislocation under sterile conditions described previously (Cho et al., 1998c). Briefly, splenocytes were released by teasing into RPMI1640 medium supplemented with 20-mM HEPES buffer. After removing red blood cells using 0.83% NH_4Cl–Tris buffer (pH 7.4), splenocytes were washed three times in Ca^{2+}–Mg^{2+}-free Hank's balanced salt solution and resuspended to 5×10^6 cells/ml in RPMI1640 with 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 10% fetal bovine serum.

2.8. Splenocyte proliferation assay

Splenocytes (5×10^6 cells/ml) were cultured in flat bottom 96 well plates in the presence and absence of various T or B lymphocyte mitogens (concanavalin A, phytohemagglutinin and lipopolysaccharide) with cynaropicrin and positive control drugs in a total volume of 200 μl/well at the same conditions for 48 h (Cho et al., 1998c). The proliferation assay was performed by MTT assay.

2.9. Interleukin-2 dependent cell proliferation assay

CTLL-2 cells were harvested from interleukin-2 containing growth medium and washed twice with RPMI 1640 without fetal bovine serum and interleukin-2. They were resuspended in growth medium without interleukin-2 to 5×10^5 cells/ml. 50-μl of cell suspension was placed into each well of a 96 well tissue culture plate and incubated in the presence of cynaropicrin and 25 U/ml of interleukin-2 for 48 h (Stanly et al., 1990).

2.10. MTT assay (colorimetric assay) for measurement of cell proliferation

Cell proliferation was measured by conventional MTT assay. At 4 h prior to culture termination, 10 μl of MTT solution (10 mg/ml in phosphate buffered-saline) was continuously cultured until termination. Culture was stopped by addition of 15% sodium dodesyl sulfate into each well for solubilization of formazan and the optical density (OD) at 570 nm ($OD_{570-630}$) was measured by a microplate Spectramax 250 microplate reader.

2.11. Cytotoxicity assay

Cytotoxicity of cynaropicrin was evaluated by MTT assay with minor modification as reported previously (Cho et al., 1998b). The cell suspension of 0.5×10^6 cells/ml was plated in 96-well plate. After 2-h culture, varying concentrations of testing compounds were added to each well and cultured for another 6, 24 and 48 h. Cell viability was measured by MTT method.

2.12. Statistical analysis

All values expressed as mean ± S.E.M. were obtained from four separate observations performed in triplicate or triplicate measurements ($n = 3$). The Student's *t*-test for unpaired observation between control and experimental samples was carried out for statistical evaluation of a difference; P values of 0.05 or less were considered as statistically significant.

3. Results

3.1. Cytotoxicity of cynaropicrin against macrophages

It was necessary to assess the cytotoxic effect of cynaropicrin before further in vitro tests were carried out. MTT assay using RAW264.7, U937 and Sup-T1 cells is a suitable model for this purpose, as the cells are representative macrophages and CD4 + lymphocytes. Fig. 2 shows that neither RAW264.7 nor U937 cells were cytotoxic for 6-h incubation at the TNF-α inhibitory concentrations suggesting that cynaropicrin may inhibit TNF-α production without any interference of normal cell function. In contrast, cynaropicrin inhibited macrophage proliferation at incubation for 24 h with the IC_{50} values of 16.3 (RAW264.7) and 17.2 (U937) μM, respectively, and suppressed Sup-T1 proliferation at incubation for 24 and 48 h with the IC_{50} values of 27.5 and 14.1 μM, respectively. Unlike cancer cell lines, cynaropicrin significantly inhibited the viability of normal splenocytes at low concentra-

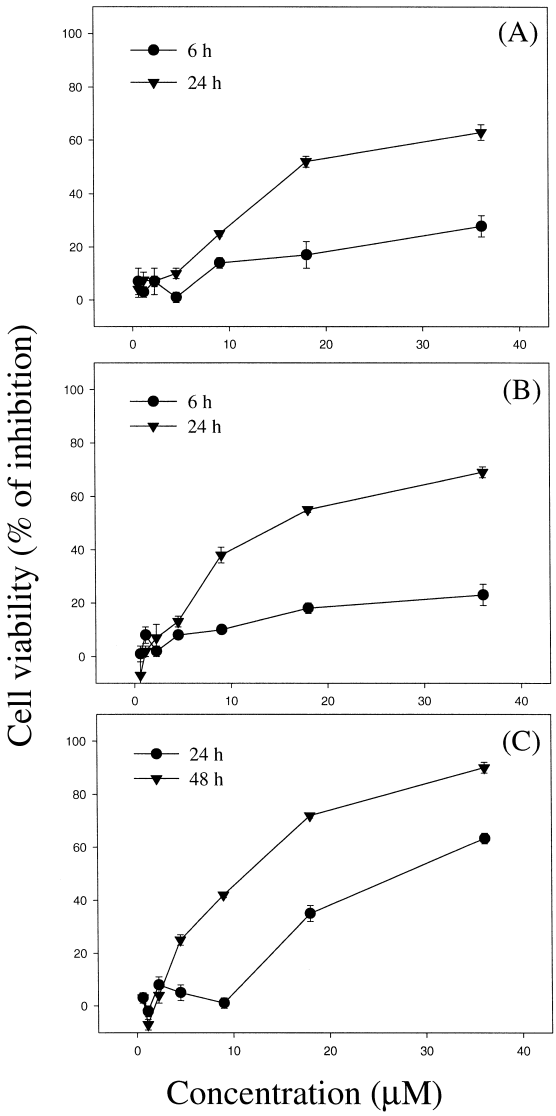

Fig. 2. Effect of cynaropicrin on the viability of U937 (A), RAW264.7 (B) and Sup-T1 (C) cells. Cell viability was assayed by conventional MTT method as described in Materials and methods. Data represent mean ± S.E.M. of four separate observations performed in triplicate.

tions suggesting that it may enhance normal apoptotic death of splenocytes under the conditions in the absence of mitogens.

3.2. Effect on TNF-α release

To compare the inhibitory effect of cynaropicrin in both murine and human macrophages, we used RAW264.7 cells and differentiated U937 cells stimulated by lipopolysaccharide. The optimal lipopolysaccharide concentration (1 μg/ml) and incubation time (6 h) for maximum TNF-α release were determined in the preliminary experiments (data not shown) and used throughout the experiments.

As shown in Fig 2 and Table 1, cynaropicrin strongly inhibited lipopolysaccharide-induced TNF-α release from either murine or human macrophage cells in a dose-dependent manner with the IC_{50} values of 8.24 and 3.18 μM suggesting that cynaropicrin inhibits TNF-α release in human as well as in murine, and its inhibitory activity is more sensitive in human macrophage U937 than murine macrophage RAW264.7. Positive control drugs, dbcAMP and prednisolone, used in this experiment also significantly suppressed TNF-α release in a dose-dependent manner with the IC_{50} values of 28.9 and 25.4 μM, respectively.

3.3. Effect on NO release

Culture supernatant of RAW264.7 cells contained 0.5 to 1 μM of NO as a basal level. When RAW264.7 cells were stimulated with 1 μg/ml of lipopolysaccharide and 50 U/ml of interferon-γ for 24 h, NO was produced about 30 to 55 μM in culture medium, more than 30- to 100-fold amount than the basal level. So, these conditions were applied to analyze suppressive effect by cynaropicrin and positive control drug, L-NAME on NO release from RAW264.7 cells stimulated by lipopolysaccharide and interferon-γ. Cynaropicrin was added simultaneously with activators and accumulation of NO was measured 24 h later (Fig. 4 and Table 1). Cynaropicrin dose-dependently inhibited NO release by both signals with the IC_{50} values of 1.10 and 1.48 μM, respectively. L-NAME also inhibited NO release from either lipopolysaccharide- or interferon-γ-stimulated RAW264.7 cells with the IC_{50} values of 193.3 and 116.2 μM, respectively.

3.4. Effect on lymphocyte proliferation

We examined the inhibitory effects of cynaropicrin on lymphocyte proliferation from splenocyte in the presence of mitogens by MTT assay. In this assay, concanavalin A

Table 1
Inhibitory effect of cynaropicrin on TNF-α and NO release from macrophages and lymphocyte proliferation

Parameter	IC_{50} (μM)
TNF-α release	
RAW264.7	8.24 ± 0.19
U937	3.18 ± 0.28
NO release	
Lipopolysaccharide	1.10 ± 0.02
Interferon-γ	1.48 ± 0.03
Lymphocyte proliferation	
Concanavalin A	1.20 ± 0.01
Phytohemagglutinin	1.02 ± 0.06
Lipopolysaccharide	0.90 ± 0.09
Interleukin-2-dependent cell proliferation	
CTLL-2	0.91 ± 0.04

Data represent mean ± S.E.M. of four separate observations performed in triplicate.

was added at a concentration of 1 μg/ml when splenocytes were cultured, and phytohemagglutinin and lipopolysaccharide were added at a concentration of 10 μg/ml as an optimal concentration. The proliferation of T- or B-lymphocytes treated by mitogens was significantly increased by three times to four times compared with untreated cells. As shown in Fig. 5 and Table 1, cynaropicrin showed significant inhibitory effects toward all mitogenic signals with the IC_{50} values of 1.20 (concanavalin A), 1.02 (phytohemagglutinin) and 0.90 μM (lipopolysaccharide), respectively. Standard drugs, A77,1726, a protein tyrosine kinase inhibitor, and L-cycloserine, an inhibitor of sphingolipid biosynthesis, in concanavalin A-stimulated splenocyte proliferation showed the IC_{50} values of 6.7 and 3.5 μM, respectively.

3.5. Effect on CTLL-2 proliferation

In order to examine the inhibitory effect of cynaropicrin on CD8 + cell proliferation, CTLL-2 cells were used as a model system. The standard curve by interleukin-2 showed a linearity ($r^2 = 0.940$) between 0.3 and 40 U/ml as a final concentration (data not shown). Cynaropicrin suppressed CTLL-2 cell proliferation in a dose-dependent manner (Fig. 6 and Table 1) and the 50% inhibitory concentration (IC_{50}) of cynaropicrin for CTLL-2 cell growth was 0.91 μM. It is similar to inhibitory effect of the compound on the mitogenic response of mouse splenocytes (Table 1). Positive control drugs, A771726 and dbcAMP, dose-dependently inhibited the proliferation with the IC_{50} values of 11.2 and 576 μM, respectively.

3.6. Effects of sulphydryl compound L-cysteine

According to previous reports that sesquiterpene lactones may covalently interact with sulphydryl groups of target protein(s) (Hwang et al., 1996; Hehner et al., 1998), in this study L-cysteine was added with cynaropicrin to the cell culture media in testing pharmacological assay. As expected, the L-cysteine (150 μM) suppressed the inhibitory effects of cynaropicrin in all experiments tested (Table 2) suggesting that binding between cynaropicrin and sulphydryl groups of target protein(s) plays a crucial role in pharmacological action of cynaropicrin regardless of cell type and signal. As a further analysis, we characterized either whether L-cysteine inhibits the pharmacological action of cynaropicrin in a dose-dependent manner or whether cynaropicrin irreversibly inhibits TNF-α.

Fig. 7A shows the inhibitory effect of L-cysteine is related to molar ratio between cynaropicrin and L-cysteine. As shown, L-cysteine inhibited the inhibitory action of cynaropicrin in a dose-dependent manner and completely abrogated at high concentration. To confirm time-dependence, we post-treated L-cysteine 1 or 2 h after cynaropicrin treatment. As the result, Fig. 7B shows that the inhibitory effect of L-cysteine is dependent on treatment time. Thus, the effect was markedly increased at the same time incubation, but largely decreased at 1 and 2 h post-treatment suggesting that cynaropicrin may tightly bind to target proteins within 2 h. We next tested whether cynaropicrin acts as competitive or irreversible inhibitors. For this, RAW264.7 cells were incubated with 9-μM cynaropicrin for 2 h as reported by Hehner et al. (1998). Subsequently, the cells were washed with medium void of cynaropicrin and further grown for 2 h in cynaropicrin-free medium. After stimulation with lipopolysaccharide for 6 h, TNF-α from culture medium was assayed by ELISA. The total inhibitory effect seen after immediate stimulation of cells following the pre-incubation with cynaropicrin was set as 100%. As shown Fig. 7C, the inhibition was still almost maintained after 2-h incubation in cynaropicrin-free medium. This result suggests that cynaropicrin acts by covalently and thus irreversibly modifying its target protein(s), presumably by its reactive Michael system in the lactone ring. In addition, there were no significant effects on combination treatment with L-cysteine and positive control drugs (prednisolone, L-NAME and A77,1726) as well as single treatment of L-cysteine in the relevant pharmacological assays (data not shown).

Table 2
Sulphydryl compound (L-cysteine) inhibits the suppressive effect of cynaropicrin on TNF-α and NO release from macrophage, and lymphocyte proliferation

Parameter	% of inhibition	
	Cynaropicrin	Cynaropicrin + L-cysteine
TNF-α release[a]		
RAW264.7	92.3 ± 4.8	26.4 ± 2.1
U937	99.6 ± 4.7	22.7 ± 3.3
NO release[a]		
Lipopolysaccharide	99.7 ± 2.9	20.8 ± 6.4
Interferon-γ	96.7 ± 3.2	28.7 ± 5.1
T cell proliferation[a]		
Concanavalin A	99.1 ± 0.3	22.7 ± 3.1
Phytohemagglutinin	96.3 ± 1.1	26.8 ± 5.1
Lipopolysaccharide	92.1 ± 8.3	28.1 ± 0.4
Interleukin-2 dependent cell proliferation[a]		
CTLL-2	98.4 ± 3.7	29.4 ± 3.6

Cells were stimulated by different stimuli in the presence of cynaropicrin (18 μM) and L-cysteine (150 μM). Data represent mean ± S.E.M. of triplicate measurements ($n = 3$).

4. Discussion

In the course of screening of natural products to find novel anti-inflammatory drugs as the TNF-α inhibitors (Chae et al., 1998; Cho et al., 1998a,b,c,d, 1999), we have shown previously that sesquiterpene lactones, including cynaropicrin, reynosin and satamarine, from *S. lappa*, suppress cytokine production in a concentration-dependent

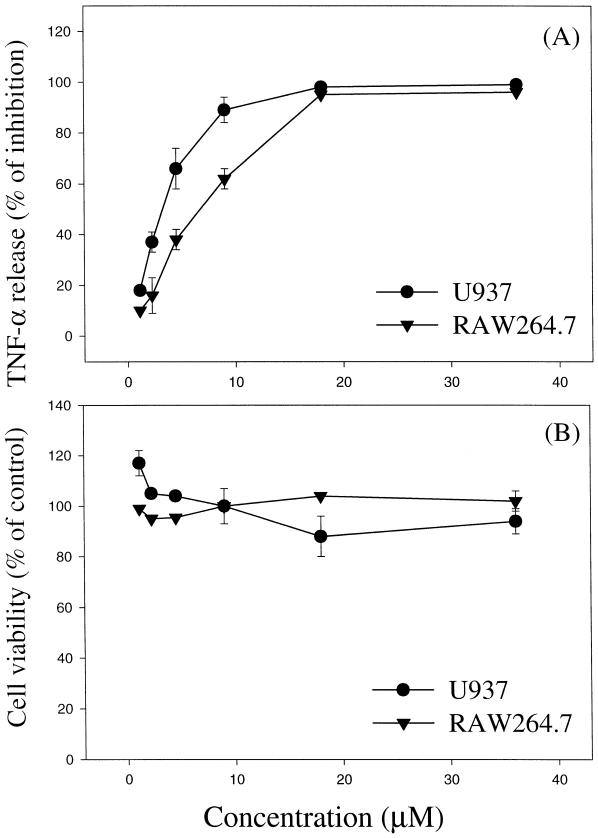

Fig. 3. The inhibitory effect of cynaropicrin on TNF-α release from RAW264.7 and differentiated U937 cells stimulated by 1 μg/ml of lipopolysaccharide for 6 h. (A) TNF-α production was assayed from culture supernatants of lipopolysaccharide (1 μg/ml)-stimulated RAW264.7 and differentiated U937 cells (1×10^6 cells/ml) in the presence of various concentrations of cynaropicrin. (B) Cell viability under the same conditions was assayed by MTT method as described in Materials and methods. Data represent mean ± S.E.M. of four separate observations performed in triplicate.

manner (Cho et al., 1998b; Jung et al., 1998). In particular, cynaropicrin strongly inhibited TNF-α release from murine macrophage stimulated by lipopolysaccharide (Cho et al., 1998b) and the inhibitory effect was abrogated by co-treatment with sulphydryl compounds (Cho et al., 1998b), as demonstrated from the pharmacological study of other sesquiterpne lactones (Hwang et al., 1996; Bork et al., 1997; Hehner et al., 1998; Lyß et al., 1998). In this study, we examined and characterized the immunoregulatory effects of cynaropicrin on the release of major inflammatory mediators such as NO or TNF-α from macrophages as well as suppressive effect on lymphocyte proliferation, as a part of evaluation of in vitro anti-inflammatory effects.

Cynaropicrin completely abolished the release of TNF-α and NO from either differentiated U937 cells or RAW264.7 cells activated by lipopolysaccharide and interferon-γ. The compound also strongly suppressed the proliferation of CD4+, CD8+ T- and B-lymphocytes treated by concanavalin A, phytohemagglutinin, lipopolysaccharide and interleukin-2. These inhibitory activities (IC_{50}) are summarized in Table 1. The effects seem to be not due to the non-specific cytotoxicity, since cynaropicrin did not affect cell viability at the pharmacologically effective doses (Figs. 2–5).

The inhibitory potency of cynaropicrin on TNF-α and NO production is considerable when compared with other natural products or clinically available drugs. The activities are similar with other sesquiterpene lactones or higher than those of previously isolated natural compounds, such as ginsenosides, flavonoids and coumarins (Hwang et al., 1996; Park et al., 1996; Chae et al., 1998; Cho et al., 1998a,b,c; Ryu et al., 1998; Lee et al., 1999), and pentoxifylline and prednisolone, suggesting that sesquiterpene lactones may highly interact with key enzyme(s) by which the production of TNF-α and NO is regulated, and may represent a novel agent for the pharmacological control of inflammation. This notion expanded us that cynaropicrin seems to possess a wide range of therapeutic activities against the TNF-α or NO-mediated diseases such as arthritis, tumor metastasis, or even AIDS, since TNF-α and NO

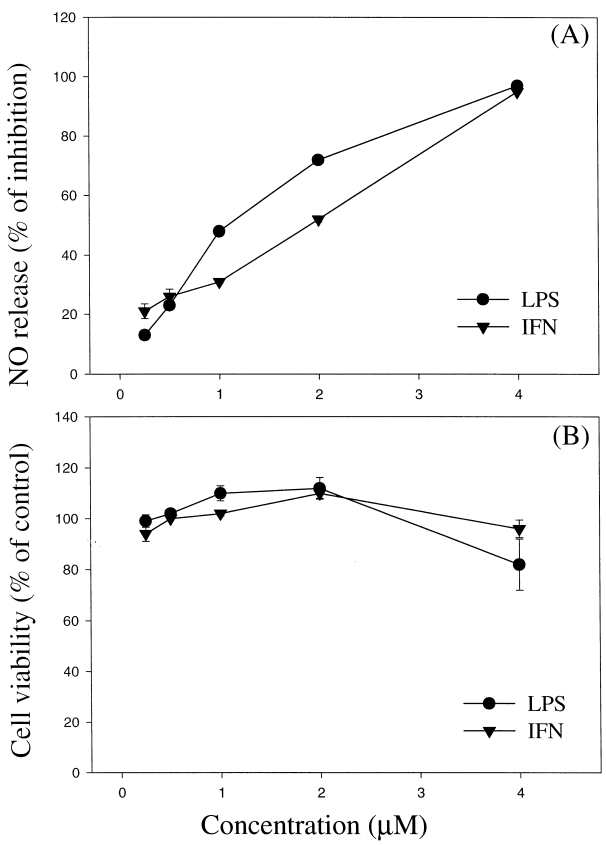

Fig. 4. Effect of cynaropicrin on NO release from RAW264.7 cells stimulated by lipopolysaccharide and interferon-γ. (A) RAW264.7 cells (1×10^6 cells/ml) were incubated with various concentrations of cynaropicrin in the presence of 1 μg/ml of lipopolysaccharide or 50 U/ml of interferon-γ for 24 h. Supernatants were collected and assayed for nitrite. (B) Cell viability under the same conditions was assayed by MTT method as described in Materials and methods. Data represent mean ± S.E.M. of four separate observations performed in triplicate.

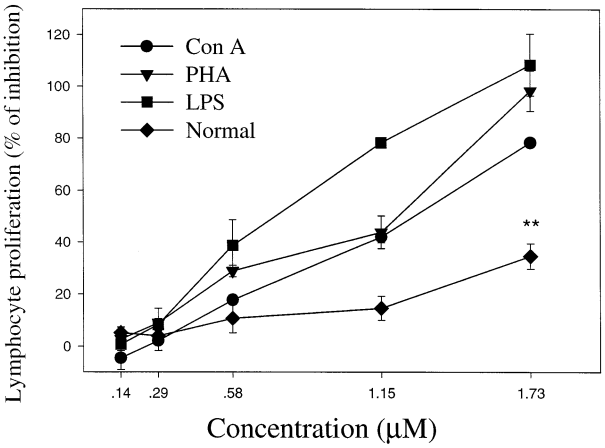

Fig. 5. Effect of cynaropicrin on lymphocyte proliferation from mouse splenocytes in response to concanavalin A, lipopolysaccharide and phytohemagglutinin. Splenocytes (5×10^6 cells/ml) were incubated with various concentrations of cynaropicrin in the presence of 1 μg/ml of Con A, 10 μg/ml of lipopolysaccharide and 10 μg/ml of phytohemagglutinin for 48 h. Cell proliferation was assayed by conventional MTT method. Data represent mean ± S.E.M. of four separate observations performed in triplicate. Basal and stimulated OD values of lymphocyte were 0.20–0.21 (basal), 1.40–1.45 (concanavalin A), 0.65–0.70 (lipopolysaccharide) and 0.80–0.85 (phytohemagglutinin), respectively. $^{**}P < 0.01$ represents significant difference compared to no treatment.

are known to play a central role in several stages of chronic inflammatory diseases and cancer, including angiogenesis, cell adhesion and release of inflammatory mediators, and in DNA replication of HIV (Beutler, 1995; Sekut and Connolly, 1996; Jackson et al., 1998).

In addition to the activation of macrophage by endotoxin or cytokine, it is known that either CD4 + or CD8 + T lymphocytes play a crucial role in chronic inflammatory processes through activation of inflammatory cells such as mast cells, eosinophils, and neutrophils, and macrophages, resulting in massive production of chemical mediators and pro-inflammatory cytokines (Panayi et al., 1992; Correll et al., 1997). By the reason, immunosuppressive agents such as cyclosporin A have been known to show anti-inflammatory properties in clinical treatment against chronic diseases. The notion allowed us to further analyze the immunosuppressive effect of cynaropicrin. In particular, to carefully examine anti-mitogenic response to lymphocyte proliferation, the inhibitory effects of cynaropicrin on mitogenic proliferation of lymphocytes and the normal proliferation of Sup-T1 cell, a representative CD4 + T lymphocyte were compared. Interestingly, our results (Figs. 2, 5 and 6) indicate that cynaropicrin suppresses mitogenic proliferation 10 times more than normal Sup-T1 cell proliferation, suggesting that the mitogenic responses may be more selective target(s) to the compound. Moreover, a similar pattern was confirmed from another experiment using other types of cells, such as eosinophils (Eol-1), normal liver cells (Chang liver cells) and fibroblasts (Cho et al., unpublished data), as shown in Sup-T1 cells. Taken together, the selective inhibition of cynaropicrin suggests that the pharmacological activities of the compound on lymphocyte proliferation are not due to non-specific cytotoxicity, as shown in TNF-α and NO production assays and cynaropicrin may act as a potent and selective immunosuppressive agent.

Although the mechanism whereby cynaropicrin inhibits the mitogenic responses to TNF-α and NO release and lymphocyte proliferation is yet to be established, the compound appears to inhibit common pathway(s) of mitogenic responses. To date, several important common biological pathways have been generally demonstrated from intensive researches. One of the pharmacological targets of cynaropicrin could be postulated as the transcriptional factor, nuclear factor (NF)-κB, since there are some reports that sesquiterpene lactones selectively suppress NF-κB activation through an indirect interaction with I-κB (Hehner et al., 1998) or a direct binding with NF-κB (Lyß et al., 1998). However, additionally, it is a possibility that cynaropicrin may non-selectively inhibit protein tyrosine phosphorylation, one of the common biochemical pathways, elicited by various kinds of mitogens and cytokines, as reported in the previous study (Hwang et al., 1996), since we have confirmed that cynaropicrin inhibited tyrosine phosphorylation induced by integrin engagement, dose-dependently (Cho et al., unpublished data). Therefore, to elucidate the exact target(s) of cynaropicrin, further studies should be added.

Despite many studies, which prove anti-inflammatory activity of sesquiterpene lactones, the molecular mechanism has not been sufficiently explained. However, it is speculated that sesquiterpene lactones may interact with sulphydryl groups of target protein(s) through covalently irreversible binding, as shown in several anti-inflammatory agents such as aspirin (Im et al., 1985; Karim et al., 1996).

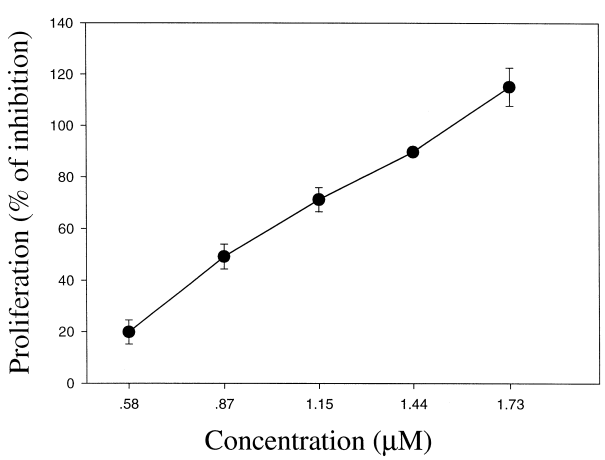

Fig. 6. Effect of cynaropicrin on CTLL-2 proliferation stimulated by 25 U/ml of IL-2. CTLL-2 (5×10^5 cells/ml) were incubated with various concentrations of cynaropicrin in the presence of 25 U/ml of interleukin-2 for 48 h. Cell proliferation was assayed by conventional MTT method. Data represent mean ± S.E.M. of four separate observations performed in triplicate. Basal and stimulated OD values of CTLL-2 cells were 0.15–0.20 and 1.00–1.10, respectively.

Indeed, the lactone ring carrying an exomethylene group forms a reactive Michael-system, which is a nucleophilic target, e.g., for sulphydryl compounds such as cysteine, dithiothreitol and 2-mercaptoethanol, as reported in several articles (Hwang et al., 1996; Bork et al., 1997; Cho et al., 1998b). Therefore, in this study to gain insight into possible molecular mechanism of cynaropicrin toward the inhibitory effect of NO and lymphocyte proliferation as well as TNF-α, L-cysteine was added to the cell culture media in testing assay. As expected, the L-cysteine (150 μM) suppressed the inhibitory effects of cynaropicrin regardless of cell type and stimuli (Table 2), suggesting that lactone ring may generally play an important role in these pharmacological activities. To further characterize not only if cysteine inhibits these effects in a dose- and time-dependent manner, but also if cynaropicrin irreversibly inhibits TNF-α release, several experiments were conducted. Fig. 7A shows that the inhibitory effect of L-cysteine is a dose-dependent manner in which more than 300 μM of cysteine (15-fold as a molar ratio between cynaropicrin and L-cysteine) attenuates the suppressive effect of cynaropicrin up to 90%, suggesting not only that high molar ratio is required to completely abrogate the cynaropicrin effect, but also that the binding affinity between cynaropicrin and target protein(s) might be higher than that between cynaropicrin and L-cysteine. Furthermore, L-cysteine effect was dependent on treatment time. The inhibitory effect of L-cysteine was clearly shown at the same time treatment, whereas L-cystein treated at 2 h after cynaropicrin and lipopolysaccharide did not inhibit cynaropicrin-induced suppression (Fig. 7B), indicating that the interaction between cynaropicrin and target protein(s) mainly occurs within 1 h up to 65% and 2 h up to 85%, respectively. Regarding irreversible mode of action, Fig. 7C displays that an irreversible mechanism such as a covalent modification is a major mode of molecular action to inhibit activation of the target(s), as reported previously (Hehner et al., 1998). Considering the mechanism, the formation of new covalently linked compound(s), which remains to be identified, between L-cysteine and cynaropicrin seems to mediate inhibitory effect of L-cysteine on the pharmacology of cynaropicrin, as shown in the case of omeprazole (Im et al., 1985). Indeed, a putative binding between cynaropicrin and L-cysteine was demonstrated from homotypic aggregation assay conducted by treatment of a mixture incubated with cynaropicrin and L-cysteine for 30 min to the cells, stimulated by CD29 antibody (Cho et al., unpublished data). In this experiment, we found that the inhibitory effect of L-cysteine on the cynaropicrin-induced suppression of homotypic aggregation of U937 cells was higher when it is pre-incubated with cynaropicrin 30 min before adding to the cells, rather than directly pre-incubated in the cells, suggesting that the lactone ring of cynaropicrin could chemically react with the sulphydryl group of L-cysteine and may attack sulphydryl group of target protein(s) to form a irreversible binding.

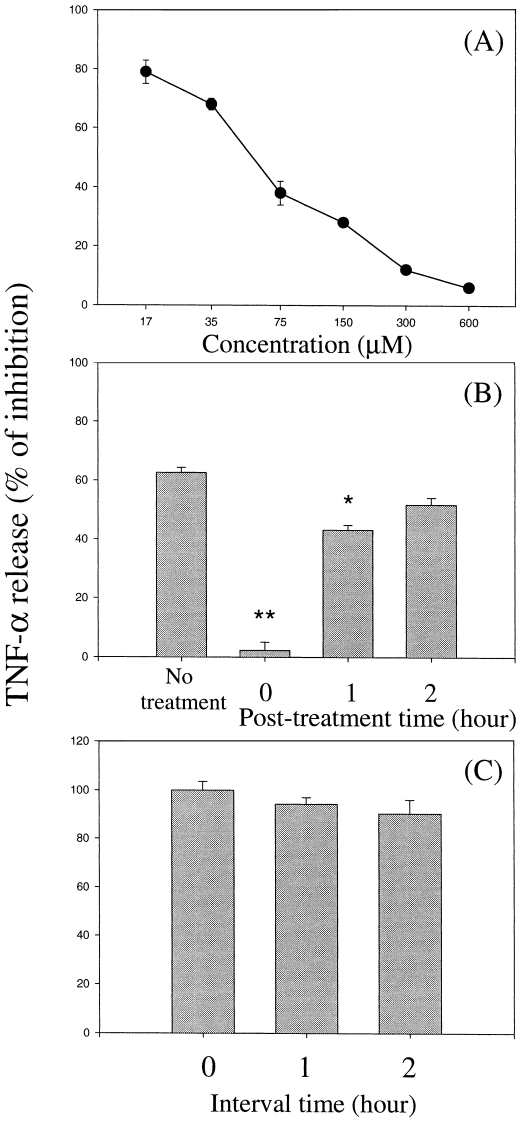

Fig. 7. Characterization of inhibitory effect of cynaropicrin using L-cysteine. (A) Dose–response of L-cysteine. RAW264.7 cells (1×10^6 cells/ml) were incubated with various concentrations of L-cysteine in the presence of cynaropicrin (18 μM) and LPS (1 μg/ml). (B) Post-treatment effect of L-cysteine. The diagram shows the percentage of inhibition of TNF-α release in dependence of the post-treatment time of L-cysteine (150 μM) in the presence of cynaropicrin (9 μM). (C) Time dependence of TNF-α inhibition. The diagram shows the percentage of inhibition of TNF-α release in dependence of the time period between cynaropicrin (9 μM) preincubation and lipopolysaccharide stimulation. The inhibition of TNF-α production without incubation in cynaropicrin-free medium was set as 100%. Data represent mean ± S.E.M. of triplicate measurements ($n=3$). $^{**}P<0.01$ and $^{*}P<0.05$ represent significant difference compared to no treatment (cynaropicrin alone).

In conclusion, we have shown that cynaropicrin dose-dependently inhibited TNF-α and NO production, and lymphocyte proliferation, suggesting a possibility that the inhibitory effects of cynaropicrin may represent an important aspect of the anti-inflammatory activities of the compound. It was also confirmed and characterized that the molecular mechanism of cynaropicrin toward these mito-

genic responses is related to interaction with sulphydryl groups of the target protein(s). Furthermore, as there have been a few reports about immunomodulatory effects of sesquiterpene lactones on the production of cytokine and inflammatory mediator, and the activation and proliferation of lymphocytes, it is regarded that these biological activities of cynaropicrin will give an important evidence to understand the anti-inflammatory effects of sesquiterpene lactones.

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