Tripterine inhibits the expression of adhesion molecules in activated endothelial cells

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Abstract: Cell adhesion molecules (CAM) expressed by vascular endothelium in response to cytokine stimulation play a key role in leukocyte adhesion to endothelium during the inflammatory response. Tripterine, a chemical compound of the Chinese plant *Tripterygium wilfordii* Hook f, displays anti-inflammatory properties in several animal models. However, mechanisms of its action are poorly understood. In the present study, we show that in inflammatory conditions, mimicked by tumor necrosis factor α (TNF-α) stimulation, pretreatment for 6 h with tripterine at nontoxic concentrations of 20–200 nM inhibits the expression of E-selectin, vascular cell adhesion molecule (CAM)-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) in human umbilical vein endothelial cells (HUVEC) in a dose-dependent manner. Tripterine (200 nM) almost completely inhibits expression of VCAM-1 [50% inhibitory concentration (IC_{50})=52 nM] and ICAM-1 (IC_{50}=51 nM) and 73% of E-selectin (IC_{50}=94 nM). This inhibition effect is prominent, compared with that of dexamethasone, ibuprofen, methotrexate, or probucol, which revealed a much weaker inhibition at doses as high as 1 mM. Effects on endothelial CAM of other proinflammatory cytokines, such as interleukin-1β and interferon-γ, were also inhibited significantly by tripterine. Moreover, significant inhibition was equally observable in postincubation experiments. In addition, tripterine inhibited adhesion of human monocytes and T lymphocytes to TNF-α-stimulated HUVEC. Finally, tripterine inhibited TNF-α-driven CAM mRNA transcription and nuclear factor-κB nuclear (NF-κB) translocation. Hence, we describe a new mechanism of tripterine's anti-inflammatory action obtained at nanomolar concentrations, owing to the negative regulation of cytokine-induced adhesion molecule expression and adhesiveness in human endothelium. *J. Leukoc. Biol.* 80: 309–319; 2006.

Key Words: *inflammation · ICAM-1 · VCAM-1 · NSAID · Chinese medicinal plant*

INTRODUCTION

Inflammation is a physiological response for body defense. However, chronic inflammation is the major cause of various severe pathologies such as rheumatoid arthritis and Crohn's disease. Inflammation relies on the adhesive interactions between circulating leukocytes and endothelial cells lining the vascular wall [1, 2]. In response to various stimuli, such as the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ), endothelial cells undergo inflammatory activation. This results in an increased surface expression of cell adhesion molecules (CAM), notably, E-selectin, vascular CAM-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). These endothelial CAM (ECAM) play a fundamental role in the process of leukocyte recruitment from the blood for tissue infiltration. Constant induction of these ECAM leads to their deregulated expression and abnormal leukocyte recruitment, as seen in chronic inflammatory disease, characterized by a profound tissue remodeling and loss of function [3]. Therefore, blocking the expression of ECAM on endothelial cells, thus inhibiting the interaction between circulating leukocytes and endothelium, may be beneficial in blunting detrimental inflammatory reactions [2, 4].

The cytokine-induced expression of ECAM is regulated by the activity of several transcription factors. In particular, the promoter regions of the genes encoding for E-selectin, VCAM-1, and ICAM-1 all have binding sites for nuclear factor (NF)-κB [5–7]. As in other cell types, NF-κB is present in the cytoplasm of unstimulated endothelial cells, and it is translocated to the nucleus following stimulation by proinflammatory cytokines. Nuclear translocation of NF-κB induces, among others, the transcription of ECAM genes. It is noteworthy that several therapeutic approaches to inflammation are based on the inhibition of the nuclear translocation of NF-κB, thus reducing the expression of ECAM [8–11].

Chemical compounds derived from plants used in traditional Chinese medicine to cure disease are important sources for the screening of new, active pharmaceutical molecules. Using this

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approach, tripterine (C_{29} H_{38} 0_4, MW 450.6), a triterpenoid from the Celastrae family, extracted from the Chinese plant *Tripterygium wilfordii* Hook f (TWHF) [12], has attracted much interest, especially in the field of anti-inflammation [13]. TWHF (whose Chinese name "Lei Gong Teng" means "thunder God vine") has been used for thousands of years in China as a remedy against arthritis and other rheumatic diseases, and its anti-inflammatory effects were proved by contemporary medicine [14]. The in vivo anti-inflammatory effects of tripterine have been demonstrated in animal models of collagen-induced arthritis [15], Alzheimer's disease [16], asthma [17], as well as in lupus-like mice [18, 19].

Nevertheless, the molecular mechanisms underlying the anti-inflammatory effect of tripterine are not yet fully understood. Several studies suggest that the relevant activity resides in the inhibition of the synthesis and secretion of proinflammatory cytokines [20, 21]. However, tripterine might also block other cytokine targets, such as the cytokine-driven expression of major histocompatibility complex II antigen [16] or inducible nitric oxide (NO) synthase, resulting in decreased NO production [22].

As mentioned above, one of the important events in inflammation is the proinflammatory cytokine-induced activation of endothelium and the resulting expression of a variety of adhesion molecules implicated in leukocyte tethering, thereby increasing their recruitment into the inflamed tissues. We made the hypothesis that tripterine might inhibit the proinflammatory, cytokine-dependent activation of endothelium. In fact, tripterine has been shown to inhibit the transcription of VCAM-1 caused by proinflammatory cytokines in murine endothelium [16]. Moreover, in some cell types, tripterine could block the nuclear translocation of NF-κB, a regulator of ECAM transcription [21].

To test our hypothesis, we examined the inhibitory effects of tripterine on the activation of human endothelium. We focused on the adherence of leukocytes to human endothelial cells activated by proinflammatory cytokines, sustained by the expression of inducible adhesion molecules. We also performed the comparison of tripterine with currently used, anti-inflammatory drugs, such as dexamethasone, ibuprofen, methotrexate, and probucol, for their efficiency in inhibiting the expression of endothelial adhesion molecules. The effect of tripterine on the nuclear translocation of NF-κB has also been evaluated. In conclusion, a new mechanism of tripterine's action is disclosed here, consisting of the inhibition of ECAM expressed during endothelial activation. This mechanism supports tripterine entirely as a potential pharmacological, anti-inflammatory molecule.

MATERIALS AND METHODS

Reagents

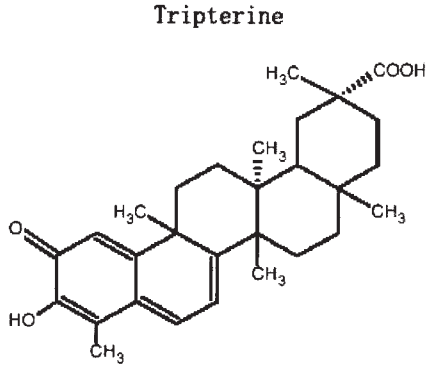
Culture media, endothelium growth medium-2 (EGM2), RPMI 1640, and EDTA-trypsin (with L-glutamine) were purchased from Gibco-BRL (Invitrogen SARL, Cergy Pontoise Cedex, France); fetal bovine serum (FBS), human fibroblast growth factor (hFGF)-B, vascular endothelial growth factor (VEGF), R^3-insulin-like growth factor-1 (IGF-1), and human epidermal growth factor (hEGF) were obtained from Cambrex Bio Science Walkersville, Inc. (Cambrex Bio Science Paris, S.a.r.l., Emerainville, France); the human recombinant (hr)TNF-α, IL-1β, and avian mylobastosis virus (AMV) reverse transcription (RT) system were purchased from Promega France (Charbonnieres); bromodeoxyuridine (BrdUrd) kit flow cytometry assay kit, R-phycoerythrin (R-PE)-conjugated mouse anti-human VCAM-1 (CD106) monoclonal antibody (mAb), R-PE-conjugated mouse anti-human E-selectin (CD62E) mAb, and R-PE-conjugated mouse anti-human CD45 mAb came from BD Bioscience-Pharmingen (Becton Dickinson France SAS, Le Pont-De-Claix Cedex); fluorescein isothiocyanate (FITC)-conjugated mouse human ICAM-1 (CD54) antibody was purchased from Diaclone Company (Besançon, France); goat anti-human NF-κB p65 polyclonal antibody and FITC-conjugated mouse anti-goat polyclonal antibody were products of Santa Cruz Biotechnology, Inc. (CA); RNeasy Mini RNA extract kit was from Qiagen S.A. (Courtaboeuf Cedex, France), 2× quantitative polymerase chain reaction (qPCR) MasterMix Plus for SYBR Green, SYBR Green, and other PCR reagents, including 18S rRNA genomic control kit, were from Eurogentec (Seraing, Belgium); and dimethyl sulfoxide (DMSO) and 2-hydroxypropyl-β-cyclodextrin, water-soluble dexamethazone, ibuprofen, methotrexate, probucol, hrIFN-γ, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) kit were purchased from Sigma Chemical Co. (Sigma-Aldrich Chimie S.a.r.l., Lyon, France). Tripterine (**Fig. 1**), provided by Professor Yang (Zhongshan Hospital, Fudan University, Shanghai, China), was extracted as described previously [23]. Except tripterine, all the reagents were stored and reconstituted according to the manufacturer's recommendations.

Human umbilical vein endothelial cell (HUVEC) isolation and culture

HUVEC were collected following normal deliveries from nonhypertensive, nondiabetic, nonsmoking women, as we described previously [24]. Cells were passaged in 25 cm^2 culture flasks in 5% CO_2 humidified air at 37°C in EGM2 supplemented with FBS (final concentration 5%, v/v), hFGF-B (0.4%), VEGF (0.1%), IGF (0.4%), R^3-IGF-1 (0.1%), hEGF (0.1%), penicillin, and streptomycin. Cells at Passages 3–5 were used for all experiments.

Drug preparation

Tripterine was dissolved in DMSO to a storage concentration of 100 mM. Water-soluble dexamethasone was dissolved in water to a stock concentration



Formula: $C_{29}H_{38}O_4$ Molecular weight: 450

Fig. 1. Chemical structure and characteristics of tripterine. Origin from root of TWHF. Alternative name is celastrol. Determination of the structure: m.p.: 198~200°C. IR cm^{-1}: 3272, 2952, 1700, 1636, 1584. UV nm: 425. EI-MS m/z: 450 (M^+, base peak), 241, 202. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.57, 1.09, 1.25, 1.28, 1.43 (each 3H, s, CH_3), 2.21 (3H, s=C-CH_3), 6.32 (1H, d, J=7.3 Hz=CH), 6.49 (1H, d, J=1.2 Hz=CH), 7.06 (1H, dd, J=7.3/1.2 Hz=CH); ^{13}C-NMR ($CDCl_3$) δ ppm: 10.5q, 18.7q, 21.5q, 28.7t, 29.3qt, 29.5t, 30.7 s, 31.1t, 31.5q, 32.4q, 33.8t, 34.5t, 36.4t, 38.4q, 39.3 s, 39.9 s, 43.1 s, 44.3 d, 45.3 s, 118.3 d, 120.4 s, 120.6 d, 127.6 s, 135.4 d, 147.0 s, 165.0 s, 172.6 s, 178.3 s, 182.4 s. Purity determination. An external standard method by high-pressure liquid chromatography with Zorbax C_{18} column as fixed phase and methanol-1% HAc (87:13) as mobile phase was adopted. The detection wavelength was 425 nm, the flow rate was 1.0 mL/min, and the purity was above 99.9%.

of 25 mg/ml. Ibuprofen, methotrexate, and probucol were dissolved with ethanol to a stored concentration of 500 mM. All were kept at –20°C, and further dilutions with culture medium were prepared before use. DMSO at proper concentration served as control for tripterine and ethanol at proper concentration as control for ibuprofen, methotrexate, and probucol; the control for water-soluble dexamethasone was 2-hydroxypropyl-β-cyclodextrin at the same concentration.

Tripterine toxicity assays on HUVEC

For toxicity assays, we determined the effects of tripterine on cell number variation by MTT assay and on cell proliferation (S-phase determination), by BrdUrd incorporation. For cell number determination, HUVEC in 96-well culture plates, at nonconfluent and confluent distribution, were incubated with tripterine at various concentrations for 24 h before performing MTT assay as detailed in the following: After incubation with the drug, HUVEC were washed twice and resupplemented with 200 μl culture medium containing 10% MTT dye. After a further 4-h incubation, the cells were washed before the addition of 100 μl DMSO. The plates were put in the dark, and continuous gentle shaking was performed for 30 min to thoroughly dissolve the MTT dye. The spectrometric absorbance at 545 nm (for formazan dye) and 690 nm (as a background level) was read using a microplate reader (Stat Fax-2100, Fisher Bioblock Scientific, Illkirch, France).

For proliferation assays, nonconfluent monolayers of HUVEC cultured in 24-well plates were incubated with tripterine at various concentrations for 24 h, and 45 min before ending the culture, the cells were loaded with BrdUrd. After 45 min of incubation with BrdUrd, the culture supernatants of the samples were collected and centrifuged to collect the floating cells, and the attached cells were detached by EDTA-trypsin treatment. The collected floating and detached cells from the same sample were mixed and washed twice with ice-cold phosphate-buffered saline (PBS). Harvested cells were fixed and permeabilized with the reagents provided in the kit at room temperature for 20 min according to the manufacturer and then washed. Following this, the cells were treated with DNase I for 1 h at 37°C to expose incorporated BrdUrd. The cells were then labeled with fluorescent anti-BrdUrd antibody for 20 min at room temperature in the dark. After washing the cells with PBS, 7-amino-actinomycin (7-AAD) was added before detection by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). The percentage of cells in S-phase was analyzed using the CellQuest software.

For these two tests, samples with proper concentration of DMSO were used as control.

Drug treatment of HUVEC

To test the effects of the drug on the expression of endothelial CAM, we carried out preincubation, as well as postincubation tests. In preincubation experiments, HUVEC were grown in 24-well culture plates to confluence and then subjected to various concentrations of tripterine or dexamethasone, ibuprofen, and methotrexate probucol (using the solvents of each drug at their respective concentrations as control) for 6 h before stimulation with TNF-α (10 ng/ml), IL-1β (5 ng/ml), or IFN-γ (100 ng/ml) for 4 h (induction of E-selectin) or 18 h (induction of VCAM-1 and ICAM-1) [25–28]. In postincubation experiments, the order of loading tripterine and cytokines was reversed, i.e., confluent HUVEC were prestimulated with TNF-α (10 ng/ml), IL-1β (5 ng/ml), or IFN-γ (100 ng/ml) for 6 h and then loaded with tripterine or a proper concentration of DMSO (control) for another 18 h. In preincubation or postincubation experiments, HUVEC without any treatment served as negative control.

To test the effects of tripterine on the expression of ECAM mRNA, nuclear translocation of NF-κB, and adhesion of some types of leukocytes, HUVEC were grown to confluence in six-well culture plates, on coverslips put into six-well culture plates, and in 24-well culture plates, respectively. Cells were then pretreated with DMSO or tripterine (200 nM) for 6 h before 1 h (for NF-κB detection) or 18 h (for transcription and adhesion assays) stimulation with TNF-α (10 ng/ml) and subjected to each particular protocol for further treatment, as detailed in the following.

Flow cytometry analysis of ECAM

The expression of three adhesion molecules, ICAM-1, VCAM-1, and E-selectin, was analyzed by flow cytometry. After being treated as described above, the HUVEC were detached by trypsine-EDTA treatment, collected, and washed twice in a cold solution of PBS containing 0.5% bovine serum albumin (BSA). Cells (5×10^5) were then resuspended in 50 μL PBS-0.5% BSA solution containing appropriate concentrations of the antibodies raised against the three adhesion molecules: mouse anti-human ICAM-1-FITC (Diaclone, 10 μL for 10^6 cells), mouse anti-human VCAM-1-PE (Becton Dickinson, 20 μL for 10^6 cells), and mouse anti-human E-selectin-PE (Becton Dickinson, 20 μL for 10^6 cells). Corresponding mouse immunoglobulin G1 isotypes labeled with FITC or PE in parallel were used as controls. After 30 min incubation at 4°C in the dark, cells were washed twice and analyzed immediately by flow cytometry. All cytometry analyses were performed on a FACSCalibur cytometer (Becton Dickinson) using the CellQuest software. A gate was established to target intact endothelial cells for adhesion molecule expression studies on the basis of adequate forward-scatter versus side-scatter (SSC) patterns.

Adhesion assay

Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood of healthy donors by implementing a standard Pancoll procedure. PBMC, Jurkat, Raji, or U937 cells (purchased from American Type Culture Collection, Manassas, VA) at 2×10^5/mL were cultured in RPMI 1640 supplemented with 10% fetal calf serum in 5% CO_2 humidified air at 37°C. The adhesion of PBMC, Jurkat, Raji, or U937 cells, coincubated with confluent HUVEC, was quantified by the ratio of leukocytes over endothelial cells by means of flow cytometric discrimination of leukocytes and HUVEC based on anti-CD45 labeling, as described in the literature [29]. Following different treatments, confluent HUVEC were washed, and 1 mL suspension of cells containing 5×10^5 PBMC, Jurkat, Raji, or U937 cells was added and incubated for 45 min at 37°C. Next, the nonadherent cells were removed by mechanical shaking of the plate for 3 min at 150 oscillations per min and gently washed three times with PBS. HUVEC, as well as PBMC or leukemic cells adhering to a HUVEC monolayer, were detached by treatment with EDTA-trypsin. Samples were labeled with anti-human R-PE-CD45, which recognizes PBMC or leukemia cells. A cytometer was set to acquire 10,000 events (HUVEC plus PBMC or leukemic cells). The number of CD45-positive cells was counted by CellQuest software, and the ratio of PBMC or leukemic cells over HUVEC was calculated.

Conventional RT-PCR detection of endothelial CAM mRNA in HUVEC

Total RNA was isolated from cultured cells by the RNeasy Mini kit. One-fifth of the total RNA obtained from each sample was used for RT in a 20-μl reaction mixture by the AMV transcription system, as indicated by the manufacturer. Incubation was carried out at 42°C for 1 h. The temperature of the reaction was then set to 95°C to inactivate the enzyme and finally lowered to 4°C. An aliquot of cDNA (2 μl) was dissolved in 50 μl of a reaction mixture containing 100 pM doexy-unspecified nucleoside 5′-triphosphates, 10 pmol forward and reverse primers, 1.5 mM $MgCl_2$, 2.5 U Taq polymerase, and 1× reaction buffer. The amplification profile consisted of an initial denaturation step at 94°C for 3 min followed by a denaturation at 94°C for 30 s, annealing at 58°C for 40 s, and extension at 72°C for 40 s. After 30 PCR cycles, extension time in the last cycle was set to 7 min, and then the PCR product was kept at 4°C. Amplification products obtained by PCR were separated electrophoretically on 1% agarose gel. Images of ethidium bromide-stained bands were photographed with a gel photo system (Fisher Bioblock Scientific).

The following sets of primers were used in PCR amplification [30]: E-selectin: sense 5′ AAA ATG TTC AAG CCT GGC AGT TCC 3′, antisense 5′ GTG GTG ATG GGT GTT GCG GTT TCA 3′; VCAM-1: sense 5′ TGA GCG GGA AGG TGA GGA GTG AGG 3′, antisense 5′ CAG GAT GGA GGA AGG GCT GAC CAA 3′; ICAM-1 sense 5′ CAC AAG CCA CGC CTC CCT GAA CCT A 3′, antisense 5′ TGT GGG CCT TTG TGT TTT GAT GCT A 3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH; primers were the same as previously published [31]): sense 5′ TCC AGA ACA TCA TCC CTG CCT CTA3′, antisense 5′ TGA GCT TGA CAA AGT GGT CGT TGA 3′.

Real-time RT-PCR quantification of endothelial CAM mRNA levels

Real-time PCR was performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). In our experiment, 18S rRNA in each HUVEC sample was amplified and detected using the 18S rRNA control kit fluorescein-tetramethylrhodamine as an internal reference. Simul-

taneously, the various ECAM genes were amplified using the primer couples described above and detected by SYBR Green fluorescence. Briefly, the cDNA of each HUVEC sample was synthesized as described in the conventional RT-PCR section. HUVEC cDNA sample (2.5 μl) was subjected to real-time PCR for amplification of ECAM genes and 18S rRNA under the same amplification conditions, although in separate wells of the same microplate. All real-time PCR reactions were carried out in triplicate, in a 25-μl reaction volume that contained 1× qPCR MasterMix Plus for SYBR Green, 400 nM each of the forward and reverse primer, 5 mM $MgCl_2$, 200 μM deoxy-adenosine 5′-triphosphate, deoxy-cytidine 5′-triphosphate, and deoxy-guanosine 5′-triphosphate, 400 μM deoxy-uridine 5′-triphosphate, 0.625 U AmpliTaq Gold, 0.25 μM Amperase uracyl N-glycosylase (UNG), and 1× TaqMan buffer. The mixture was incubated for 2 min at 50°C to enable UNG cleavage. AmpliTaq Gold was then activated by incubation for 10 min at 95°C. The subsequent thermal profile for SYBR Green real-time RT-PCR was 50 cycles of 95°C for 15 s and 60°C for 1 min. For each PCR, a standard curve was produced using six successive 1:10 dilutions of an ECAM-positive HUVEC sample. The relative amounts of mRNA for each tested ECAM gene in the samples were calculated by comparison with this standard curve. Results were normalized using the corresponding 18S rRNA value of the calibrator curve to perform a final ratio between the 18S rRNA content and ECAM mRNA expression in each sample [32].

Confocal microscopy observation and quantification of NF-κB nuclear translocation

After drug treatment, HUVEC cultured on coverslips were washed with PBS and fixed with precooled polyformaldehyde at 4°C for 20 min before two washings with PBS. Cells were labeled with goat anti-human NF-κB component p65 in solution containing 0.01% saponin for 2 h at 4°C. After three successive washings with PBS, FITC-conjugated mouse anti-goat secondary antibodies were added and incubated for 40 min at 4°C in the dark. At the end of the experiment, the cells were washed and covered with the fluorescent preservative reagent Mowiol containing propidium iodide (PI; for nuclear staining). The coverslips, covered by a cell monolayer, were reversed and mounted on a slide. Observations were carried out by confocal microscopy (Leica, Wetzlar, Germany).

The intensities of green light (NF-κB staining, arbitrary units) in cell cytoplasmic and nuclear areas were detected by Mivnt cellular image processing software (ShineTech Company, Shanghai, China). Specifically, nuclear areas were determined by the PI staining. The percentage of NF-κB distribution in the nuclear versus whole cell area was calculated using the following equation: percent of NF-κB labeling in nuclear area = (intensities of green light in the nuclear area−background in the nuclear area)/(intensities of green light in the whole cell−background in the whole cell) × 100.

Statistical analysis

Student's *t*-test was used to test for statistical significance ($P<0.05$ indicating significance) of differences in CAM fluorescence intensities detected by flow cytometry and in the ratio of PBMC over HUVEC in adhesion experiments.

RESULTS

Concentration-dependent effects of tripterine on the viability and proliferation of HUVEC

With confluent HUVEC, MTT assays showed that incubation with tripterine for 24 h at concentrations lower than 1 μM had no effect on the number of living cells. In contrast, at concentrations above 1 μM, tripterine began to reduce the number of living cells (detected as a 10% reduction of the relative light absorbance in this assay; **Fig. 2A**). The loss of cells was partially a result of apoptosis, as some of the dead cells were Annexin V-positive, and PI staining was negative (data not shown). However, in nonconfluent, proliferating HUVEC, the reduction of cell number began at doses above 600 nM (Fig. 2A), i.e., lower than that observed for confluent cells. One of the differences between nonconfluent and confluent cells is that the former are capable of proliferation, and the latter cease to be. Thus, the greater sensitivity of nonconfluent HUVEC toward tripterine might be a result of an inhibitory effect on

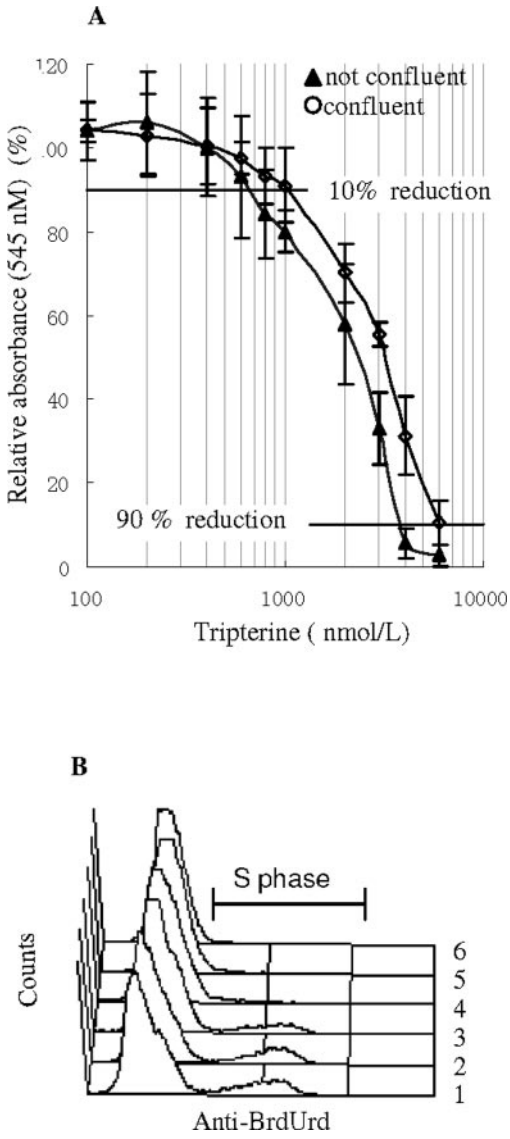


Fig. 2. Concentration-dependent effects of tripterine on cell survival and proliferation. (A) The number of viable HUVEC after incubation for 24 h with tripterine at indicated concentrations was determined by MTT assay. Cell counts of HUVEC before or after reaching confluence were reduced (defined by 10% reduction of absorbance of light) by tripterine at concentrations exceeding 600 nM and 1000 nM, respectively. The relative absorbance of drug samples was calibrated on DMSO control, set as 100%. Data points represent the mean value of three separate experiments performed in triplicate. (B) The effects of tripterine on the proportion of S-phase cells in the nonconfluent HUVEC cell cycle were determined by flow cytometry based on BrdUrd incorporation. HUVEC were incubated with medium containing DMSO alone or tripterine for 24 h. Forty-five minutes before the end of the culture, BrdUrd was added. The harvested cells were fixed and permeabilized, labeled with anti-BrdUrd antibody, stained with 7-AAD, and detected by flow cytometry. The numbers in the panel represent: 1, DMSO (with percentage of cells in S-phase 16.1%); 2–6: samples with tripterine at 400 nM (16.9%), 600 nM (9.1%), 800 nM (1.9%), 1000 nM (0.7%), and 1200 nM (0.4%), respectively.

proliferation by tripterine. This was confirmed by a dose-dependent reduction of the cell populations in S-phase caused by tripterine at concentration exceeding 600 nM, as proved by BrdUrd labeling (Fig. 2B). Therefore, the dose of tripterine used in all of the following experiments was below 200 nM to exclude toxicity.

Inhibitory effects of tripterine on ECAM expression stimulated by TNF-α

In the absence of any stimulation, the surface expression of E-selectin, VCAM-1, and ICAM-1 in HUVEC was weak, and tripterine alone showed no detectable effects on the three ECAM expressions in unstimulated HUVEC. Following 4 h stimulation with TNF-α (10 ng/ml), surface expression of E-selectin on HUVEC was induced dramatically, and higher inductions of VCAM-1 and ICAM-1 were detected following a stimulation of 18 h. Pretreatment with tripterine for 6 h inhibited the effects of TNF-α on the induction of these three ECAM in a concentration-dependent manner, as determined by flow cytometry (**Fig. 3, A–C**). The inhibitory effects of tripterine were detected at concentrations as low as 20 nM, and 73% of E-selectin and more than 90% of VCAM-1 and ICAM-1 expression were suppressed by 200 nM tripterine. The inhibitory effects were more prominent for VCAM-1 and ICAM-1 than for E-selectin, in that the 50% inhibitory concentrations for VCAM-1, ICAM-1, and E-selectin were 52 nM, 51 nM, and 94 nM, respectively (Fig. 3D).

Inhibitory effects of tripterine treatment on ECAM expression in HUVEC following stimulation with IL-1β and IFN-γ

Aside from TNF-α, other cytokines, notably, IL-1β and IFN-γ, are also involved in inflammation. We therefore investigated the effects of tripterine on these two cytokines. IL-1β (5 ng/ml) strongly induced the three ECAM, and IFN-γ (200 ng/ml) was less potent and even showed no effect on E-selectin. Preincubation with 100 or 200 nM tripterine for 6 h significantly inhibited the effects of the two cytokines (**Table 1**). The inhibitory effect of tripterine was marked in HUVEC stimulated with IL-1β as compared with those stimulated with IFN-γ. Consistent with what was observed with TNF-α, the inhibitory effect displayed by tripterine following IL-1β stimulation was more relevant for VCAM-1 and ICAM-1 than for E-selectin.

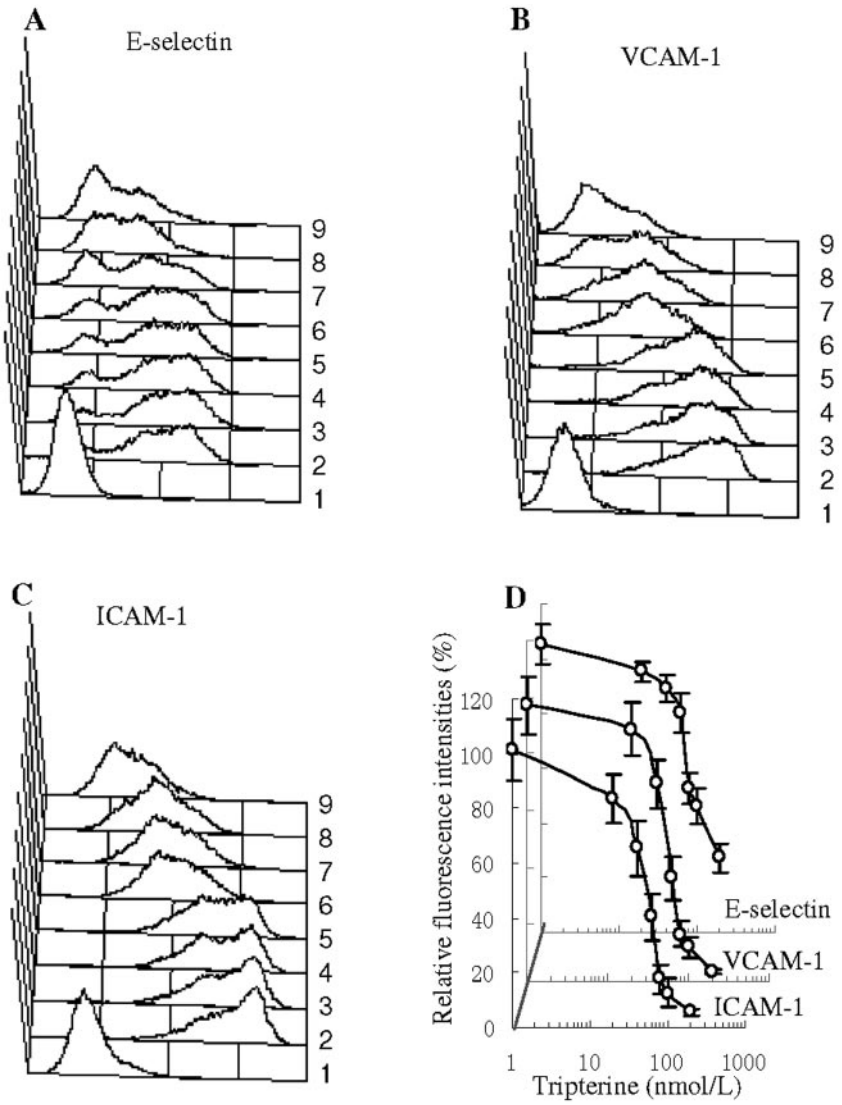


Fig. 3. Concentration-dependent inhibition by tripterine of the expressions of ECAM in HUVEC stimulated by TNF-α. The cells were treated with culture medium only or subjected to different treatments at the indicated concentrations (medium containing DMSO as control, at the corresponding concentration of the drug) for 6 h and then stimulated by TNF-α for 4 h (E-selectin) or 18 h (ICAM-1 and VCAM-1). (A–C) Respective histogram plots of flow cytometric detection of E-selectin, VCAM-1, and ICAM-1 expression in HUVEC pretreated with tripterine. In each panel, the order of plots is as follows: 1, culture medium only; 2, pretreatment with DMSO before stimulation with TNF-α; 3–9, pretreatment with 1 nM, 20 nM, 40 nM, 60 nM, 80 nM, 100 nM, and 200 nM tripterine, respectively, before stimulation by TNF-α. Each panel represents three independent repeats. (D) Relative fluorescence intensities of ICAM-1, VCAM-1, and E-selectin detected by flow cytometry in HUVEC. The relative fluorescence intensities were calculated by comparison with the sample stimulated with TNF-α after pretreatment with DMSO, the fluorescence intensity of which was set as 100%. Each point represents the mean value from three independent experiments.

TABLE 1. Inhibitory Effects of Preincubation with Tripterine on Three ECAM Expressions Detected by Flow Cytometry in Samples Stimulated with IL-1β and IFN-γ (n = 3)

	Fluorescence intensity (mean ± SD)		
	E-selectin	VCAM-1	ICAM-1
Unstimulated	7 ± 1.2	6 ± 1.3	6 ± 6.1
IL-1β-stimulated	225 ± 9.0	229 ± 46.2	248 ± 12.6
100 nM tripterine	165 ± 12.1**	46 ± 14.3***	55 ± 12.6***
200 nM tripterine	118 ± 21.4**	21 ± 3.6*	25 ± 4.8***
IFN-γ-stimulated	7 ± 0.5	22 ± 2.6	24 ± 2.1
100 nM tripterine	8 ± 0.6	12 ± 1.0**	14 ± 1.4**
200 nM tripterine	8 ± 1.0	10 ± 0.9**	12 ± 1.8**

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the samples stimulated with cytokines but not pretreated with the drug.

Postincubation inhibitory effects of tripterine on VCAM-1 and ICAM-1 in HUVEC prestimulated by three inflammatory cytokines

In clinics, when a drug is administrated to a patient during an acute episode of rheumatic disease, the inflammation is already established, and endothelium is already activated by cytokines. Thus, activation of endothelium far precedes drug application. Therefore, to approach such a situation, we added tripterine on cells that were already activated by cytokines. In tripterine postincubation experiments, HUVEC were first incubated with cytokines for 6 h and thereafter, with tripterine for another 18 h. At a concentration of 200 nM, tripterine inhibition of TNF-α-stimulated expression of VCAM-1 and ICAM-1 was lower than that obtained in preincubation assays but yet still significant with an inhibition of over 70%. The inhibitions of VCAM-1 and ICAM-1 observed following stimulation by IL-1β and IFN-γ were similar in pre- and postincubation experiments (**Table 2**). As the postincubation experiments lasted beyond the peak of ECAM induction, the expression of VCAM-1 and ICAM-1 measured was lower than those detected in the preincubation experiments. Moreover, E-selectin expression had already faded after 6 h TNF-α stimulation, and with an additional 18 h (postincubation), it decreased to the unstimulated level, even in the absence of treatment with tripterine (data not shown). Nevertheless, despite the expression shift as a result of longer incubation time, the postincubation experiment clearly showed an inhibitory effect of tripterine on ICAM-1 and VCAM-1.

Inhibitory effects of tripterine on the adhesion of leukocytes to TNF-α-activated endothelial monolayers

Theoretically, inhibition of expression of ECAM should reduce the adhesive properties of the endothelial cell monolayers, leading to a reduction of leukocyte recruitment. We thus tested this possibility by measuring the adhesion of leukocytes put in the presence of an activated endothelial monolayer. The leukocyte common antigen CD45 is expressed by PBMC and several leukemia cell lines such as Jurkat, Raji, and U937 cells, but not by HUVEC. Based on this marker, the leukocyte and HUVEC populations put together in coincubation systems were easily discriminated by flow cytometry (**Fig. 4A**) after labeling with anti-CD45 antibody. The capacity of HUVEC to tether PBMC in a 45-min coculture system was increased significantly by 18 h stimulation with TNF-α (10 ng/ml) and was partially but significantly inhibited by preincubation of HUVEC with 200 nM tripterine for 6 h. The adhesion of Jurkat (T lymphoblastic lineage), Raji (B lymphoblastic lineage), and U937 (monocytic lineage) cells on HUVEC monolayers was also increased significantly by TNF-α stimulation of HUVEC (Fig. 4B). Again, pretreatment with tripterine inhibited the increased adhesion of these cells. The inhibitory effect of tripterine was more pronounced with Jurkat and U937 cells than with Raji cells: Increased adhesion of Jurkat and U937 was almost completely suppressed by tripterine, whereas adhesion of Raji cells was not inhibited significantly. These interesting results require further investigation.

Tripterine inhibited the transcription of ECAM mRNA induced by TNF-α

We performed conventional and real-time qRT-PCR to observe the effects of tripterine on the expressions of ECAM mRNAs induced by TNF-α (**Fig. 5**).

Regarding E-selectin, 4 h and 18 h of stimulation by TNF-α showed a marked induction of the corresponding mRNA expression, although more pronounced after 4 h than 18 h. However, 6 h of pretreatment by tripterine induced a dramatic inhibitory effect on TNF-α stimulation at 4 and 18 h. Indeed, for VCAM-1 and ICAM-1, induction of their respective mRNA expressions was equally observable after 4 and 18 h of stimulation by TNF-α, although more sustained at 18 h. Again, a 6-h pretreatment with tripterine showed a considerable reduction of ECAM mRNA expression after 4 or 18 h stimulation by TNF-α.

To further confirm the inhibiting effects of tritperine highlighted above, we then performed a real-time qRT-PCR for ICAM-1 with 18S rRNA as an internal standard. The expression intensity of ICAM-1 following 4 h TNF-α stimulation was 93.5-fold that of the DMSO sample, whereas 6 h of pretreatment with tripterine reduced it to 26.9-fold (inhibition rate of 72%). With 18 h of stimulation by TNF-α, the expression of

TABLE 2. Inhibitory Effects on VCAM-1 and ICAM-1 Expressions after Postincubation with Tripterine in Samples Prestimulated with Cytokines as Detected by Flow Cytometry (n = 3)

	Fluorescence intensities (mean ± SD)	
	VCAM-1	ICAM-1
Unstimulated	5 ± 0.9	6 ± 1.4
Prestimulated by TNF-α	125 ± 25.0	456 ± 37.5
100 nM tripterine	27 ± 11.8***	163 ± 12.2***
200 nM tripterine	22 ± 15.9***	137 ± 7.6***
Prestimulated by IL-1β	40 ± 0.8	184 ± 30.2
100 nM tripterine	14 ± 1.0***	80 ± 1.2**
200 nM tripterine	8 ± 0.4***	59 ± 4.3**
Prestimulated by IFN-γ	16 ± 1.0	40 ± 3.7
200 nM tripterine	8 ± 0.3***	23 ± 3.7**

** $P < 0.01$; *** $P < 0.001$, compared with the samples stimulated with cytokines but not pretreated by drugs.

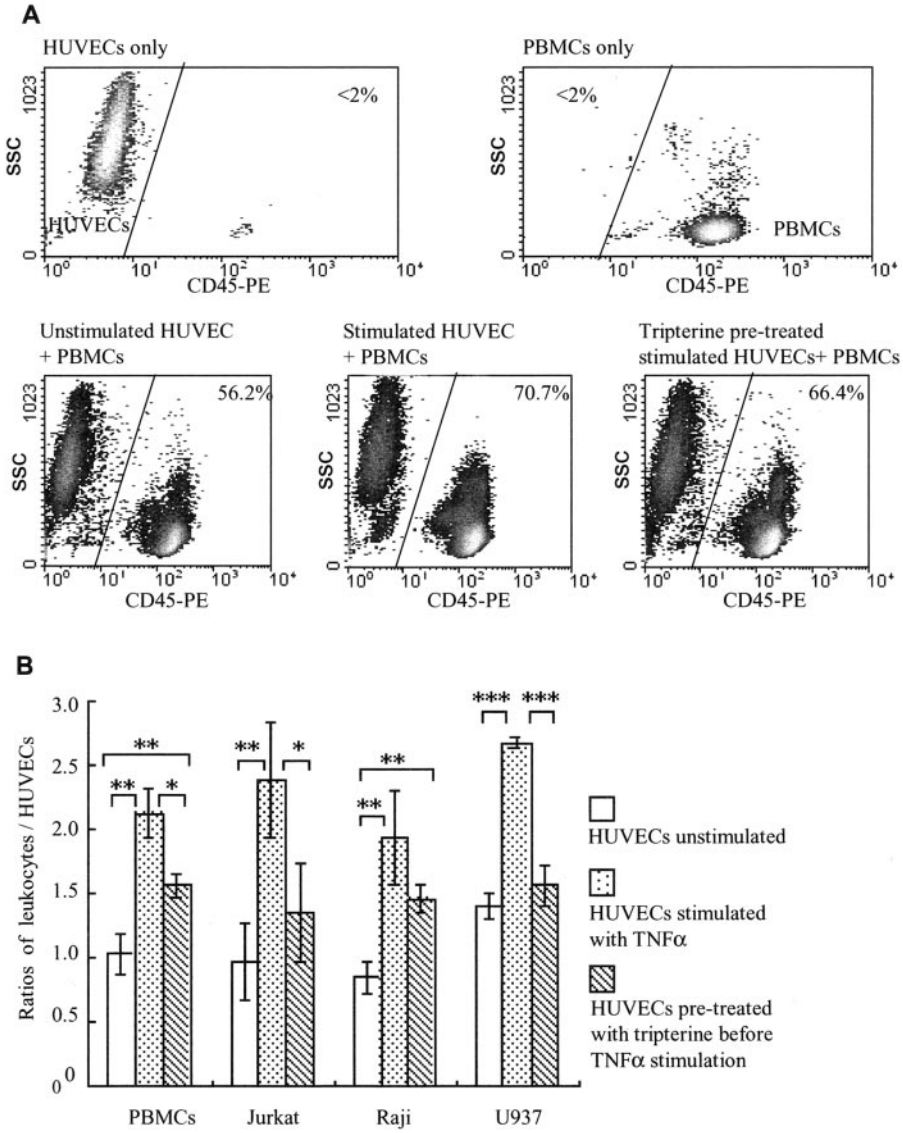


Fig. 4. Effects of tripterine on the adhesive interactions between stimulated HUVEC and leukocytes. (A) Dot plots of flow cytometric discrimination of HUVEC and PBMC after 45 min of coincubation and washing, based on the labeling with anti-CD45 antibody. The Jurkat, Raji, and U937 cells were CD45-positive and could therefore be discriminated from HUVEC in the same way as for PBMC. (B) The statistical results of the ratio of leukocytes over HUVEC after coincubation for 45 min. Each bar represented the mean of three independent tests (mean±SD, n=3). For each kind of leukocytes observed, before they were subjected to coincubation, HUVEC were incubated with culture medium only (open bars), pretreated with medium containing DMSO at a proper concentration for 6 h and then subjected to 18 h stimulation with TNF-α (dotted bars), or pretreated with 200 nM tripterine for 6 h before stimulation with TNF-α for 18 h (hatched bars). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ICAM-1 increased even further, at 349.5-fold that of the DMSO sample. The 6-h pretreatment of tripterine lowered it to 16.4-fold (inhibition rate 95.5%). Tripterine alone had only a negligible effect on the basal expression of ICAM-1 (2.8-fold modulation of intensity compared with the control).

Blockade of NF-κB translocation by tripterine

To address the possible mechanism of action by which tripterine inhibits the TNF-α-mediated ECAM overexpression, HUVEC were investigated for the nuclear translocation of NF-κB. The latter was located mainly in the cytoplasm in unstimulated cells. The percentage of NF-κB-labeling intensity in the nuclear versus total cell area accounted for 14.5 ± 3.8% (mean±SD, n=4; mean of four microscopic fields, each with at least five cells analyzed). However, when investigated after 1 h of stimulation by TNF-α, multiple NF-κB complexes showed a nuclear translocation, as the percentage of NF-κB-labeling intensity in the nuclear area increased up to 52.6 ± 5.8% (mean± SD, n=4), $P = 0.00003$. Pretreatment of tripterine for 6 h was shown to efficiently antagonize this nuclear translocation of NF-κB, as the percentage of NF-κB-related fluorescence in the nuclear area was decreased to 22.2 ± 9.4% (mean± SD, n=4), $P = 0.00151$. It is interesting that tripterine itself had no effect on the intracellular distribution of NF-κB, as the percentage of NF-κB-labeling intensity in the nuclear versus total cell area accounted for 18.0 ± 5.0% (mean± SD, n=4), $P = 0.17600$ (**Fig. 6**).

Comparison between tripterine and a panel of drugs in inhibiting TNF-α-stimulated expression of ECAM

As shown in previous parts of Results, tripterine at nanomolar concentrations dramatically inhibited adhesion molecule expression on endothelial cells stimulated with TNF-α. Thus, using the same model of TNF-α-stimulated HUVEC, the effects of a panel of drugs currently used as anti-inflammatory treatments were assessed in regard to their capacity to inhibit adhesion molecule expression in HUVEC. For E-selectin, probucol and ibuprofen showed inhibitory effects at 1 mM. Probucol also showed a significant inhibition at concentrations of 400 μM (data not shown). In contrast, dexamethasone and methotrexate had no detectable, inhibitory effect on E-selectin

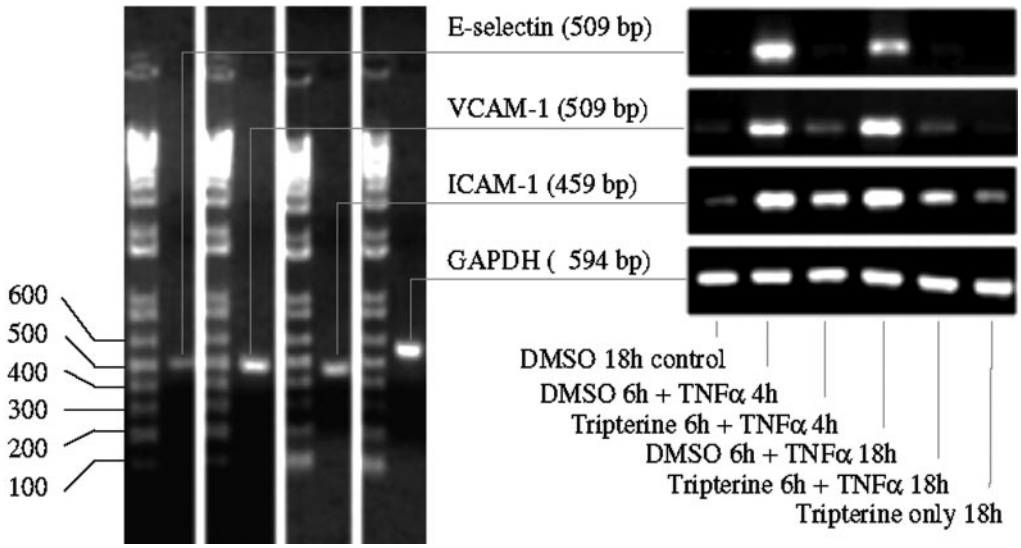


Fig. 5. Tripterine inhibition of ECAM mRNA expression in HUVEC stimulated by TNF-α. HUVEC with different treatments were harvested and subjected to RNA extraction. Conventional and real-time PCR were performed after RT of extracted RNA. The products of conventional PCR for ECAM and GAPDH are shown with ethidium bromide staining after electrophoresis in 1% agarose gel. In the left panel, each pair of primers produced specific bands with sizes as predicted. In the right panel, the results of the amplification obtained from samples with different treatments are displayed, from left to right, as follows: 18 h of treatment with DMSO; 6 h of pretreatment with DMSO plus 4 h of stimulation with 10 ng/ml TNF-α; 6 h of pretreatment with 200 nM tripterine plus 4 h of stimulation with 10 ng/ml TNF-α; 6 h of pretreatment with DMSO plus 18 h of stimulation with 10 ng/ml TNF-α; 6 h of pretreatment with 200 nM tripterine plus 18 h of treatment with 10 ng/ml TNF-α; stimulation of cells treated with 200 nM tripterine alone for 18 h.

expression, even at concentrations as high as 1 mM. For VCAM-1, dexamethasone, ibuprofen, and probucol showed inhibitory effects at concentrations of 1 mM. This effect was also significant at doses of 200 μM and 400 μM for dexamethasone and ibuprofen, respectively (data not shown). Methotrexate had no effect on VCAM-1 expression, even at 1 mM. Finally, for ICAM-1, only ibuprofen had an inhibitory effect at 1 mM (**Fig. 7**). Thus, in this experiment, tripterine was the only molecule being active on all ECAM, at a concentration as low as 200 nM.

DISCUSSION

During the inflammation process, the activation of endothelium by proinflammatory cytokines is a crucial step, as it is directly responsible for the recruitment of the circulating leukocytes and their uptake into the inflamed tissue. This process is self-maintaining, and consequently, pharmacological agents that display a smothering effect on the activation of endothelium should be efficient in anti-inflammatory activity. Here, we

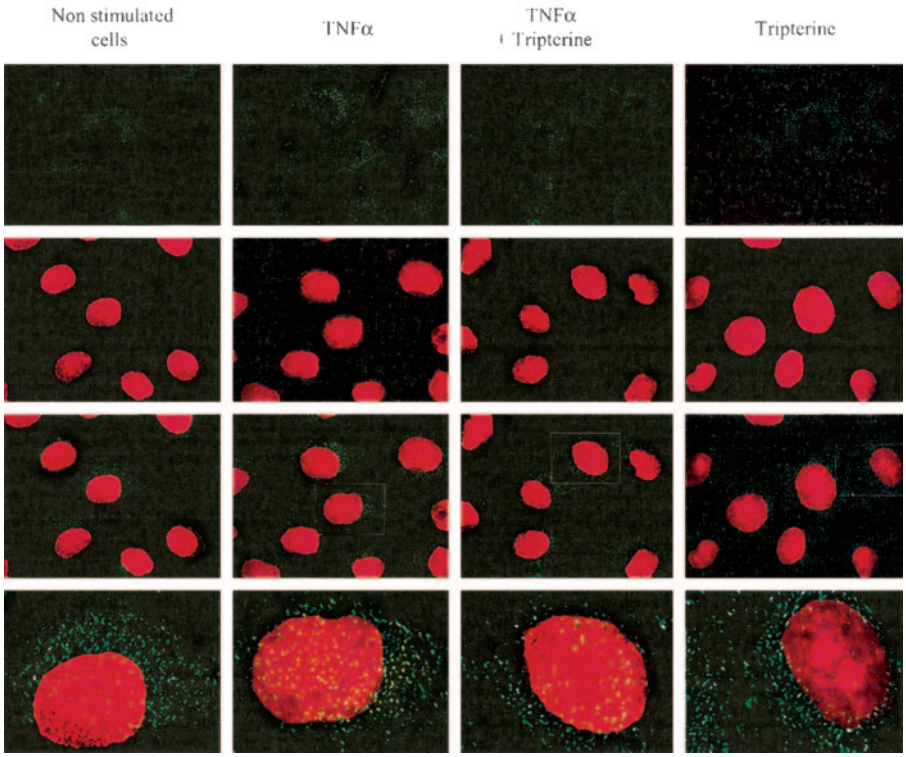


Fig. 6. Blocking of NF-κB translocation by tripterine. HUVEC were cultured on coverslips with culture medium only (control) or pretreated with DMSO or tripterine (200 nM) and then stimulated by TNF-α for 1 h. The cells were fixed and permeabilized, and labeling was performed, first, with an anti-human NF-κB antibody and then, with a FITC-labeled secondary antibody. The nucleus was stained with PI. Eventually, the coverslips were loaded with Mowiol, reversed, and mounted on a slide. The results were observed by implementing fluorescent confocal microscopy, in which NF-κB labeling appears green (top row) and nuclear staining, red (second row from top). Codistribution of NF-κB and nuclear pattern was shown in the third row from the top, and the bottom row is the magnified view of a particular cell indicated in the third row.

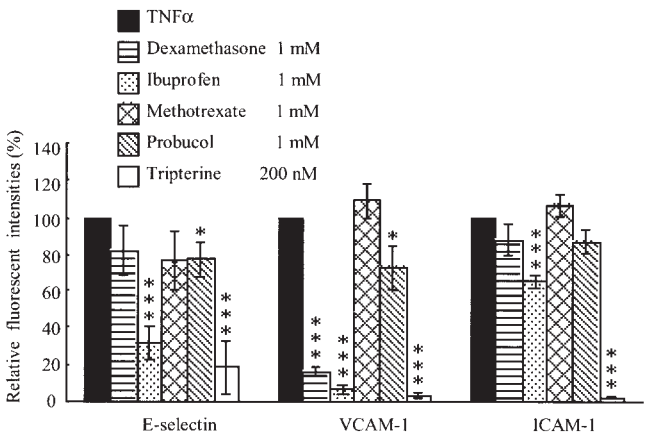


Fig. 7. Comparison between tripterine and a panel of anti-inflammatory drugs on the expression of ECAM in HUVEC stimulated by TNF-α as detected by flow cytometry. HUVEC were pretreated with the panel of indicated drugs (medium with corresponding concentration of ethanol as control) or tripterine (with DMSO as control) at the indicated concentrations for 6 h and then subjected to TNF-α stimulation for 4 h (E-selectin) or 18 h (for ICAM-1 and VCAM-1). The relative fluorescence intensities were calculated by calibrating the positive controls (stimulation with TNF-α following pretreatment with control medium containing DMSO or ethanol), the values of which were set as 100%. *, $P < 0.05$; ***, $P < 0.001$; n = 3.

provide new evidence that tripterine can inhibit the activation of endothelial cells.

Tripterine is extracted from the Chinese plant TWHF, which has been used for thousands of years in China as a remedy against arthritis and other diseases, now identified as autoimmune. In this study, we show that tripterine at nontoxic doses displays a biological activity interfering with the inflammatory activation of vascular endothelium. We have shown that below 600 nM, tripterine did not interfere with HUVEC viability. However, at doses exceeding 600 nM, tripterine inhibits cell proliferation, and toxicity becomes apparent above 1 μM. The cytostatic/cytotoxic effect of tripterine may find its application in the anti-tumor field [33–35]. However, in the present study, we have focused on the anti-inflammatory effects of tripterine at nontoxic doses. For this reason, the maximal concentration used in our study was 200 nM.

Tripterine efficiency in inhibiting E-selectin, VCAM-1, and ICAM-1 expression was observed initially in experiments in which HUVEC were preincubated with increasing concentrations of tripterine for 6 h before stimulation with TNF-α. In these conditions, the inhibitory effect was observable at doses as low as 20 nM, and at 200 nM, the inhibition was more than 90% for VCAM-1 and ICAM-1 and 73% for E-selectin. As revealed by this model, the potency of tripterine at nontoxic doses in inhibiting ECAM induction provides a new, possible explanation for its anti-inflammatory actions already observed in animal models [15–19].

During an inflammatory episode, apart from TNF-α, other proinflammatory cytokines such as IFN-γ and IL-1β also induce CAM expression in the endothelium. In this study, we show that pre- and postincubation with tripterine is equally effective to block the expression of endothelial CAM induced by theses two cytokines. Thus, it is likely that inhibiting the induction of ECAM by cytokines is a key mechanism of action in the anti-inflammatory effects of tripterine. The fact that this effect was observed with more than one cytokine enforces this hypothesis.

The postincubation tests further demonstrated the potency of tripterine in countering the effects of cytokines. Eventually, these results indicate that tripterine could be useful to treat acute episodes of inflammatory diseases.

We then speculated that one of the functional consequences of the inhibition of surface expressions of ECAM could be the smothering of the increased adhesiveness of leukocytes to activated endothelium. Confirming this hypothesis, we showed that the adhesion of human PBMC or leukemic cell lines to endothelial cells was increased by stimulating the latter, and pretreatment with tripterine could effectively inhibit this augmentation in adhesion. It is interesting to note that these inhibitory effects are dependent on the cell type. The inhibitory effect was maximal on cells with a monocytic phenotype (U937), still significant on cells with a T lymphocytic phenotype (Jurkat), and not significant on cells with features of B lymphocytes (Raji). Further investigation is needed to clarify the observed differences in the activity of tripterine toward monocytes and T and B lymphocytes, respectively. However, as T lymphocytes and monocytes play a fundamental role in regulating inflammation, the results of our functional assay provide further evidence that the anti-inflammatory action of tripterine consists essentially of the inhibition of the activation of endothelium.

We also undertook to clarify the possible mechanisms by which tripterine inhibits the ECAM overexpression described. Moreover, it was reported that during cytokine-induced expression of adhesion molecules, an important involvement of the NF-κB signaling pathway was observable [5–7]. Tripterine had already been shown to inhibit NF-κB nuclear translocation in monocytes [21]. Here, we propose that in our endothelial model, the blockage of the NF-κB nuclear translocation may account, at least in part, for tripterine's inhibitory effect on ECAM gene expression. First, the transcription of ECAM genes is NF-κB-dependent. Second, we showed that tripterine inhibits ECAM gene transcription. Third, pretreatment with tripterine inhibits the NF-κB nuclear translocation induced by TNF-α. Yet, given that we observed a potent effect with the postincubation protocol a fortiori, additional intracellular mechanisms are involved in tripterine's activity.

Although here we focused on endothelium, the two major effectors of inflammation, vascular endothelium and most circulating leukocytes, are activated by cytokines in a NF-κB-dependent signaling pathway [36]. In addition to the blocking of cytokine-driven NF-κB nuclear translocation in endothelium disclosed here, tripterine might block NF-κB nuclear translocation in leukocytes stimulated by cytokines, as shown in monocytes [21]. Thus, tripterine may achieve its potent, anti-inflammatory action on both cell types concomitantly.

Finally, the inhibitory effect of tripterine was particularly striking when compared with other well-known, anti-inflammatory drugs such as methotrexate, dexamethasone, probucol, and ibuprofen. In the same experimental conditions, i.e., HUVEC stimulated by TNF-α, tripterine was the only molecule being

active on all ECAM, at a concentration as low as 200 nM. The other three drugs could only inhibit one or two of the three ECAM but at doses at least one thousand times that of tripterine. It is interesting that we observed that at the dose of 1 mM, methotrexate increased the CAM expression in HUVEC, thus potentially displaying a proinflammatory effect. This indicates that among molecules displaying anti-inflammatory effects, tripterine is most specifically targeting activated endothelium. This specific effect places tripterine as a potential a new chemical entity in anti-inflammatory activity.

In this context, as tripterine and the anti-inflammatory drugs tested act on different targets, they could be combined to achieve a higher anti-inflammatory action. Indeed, TWHF, from which tripterine is extracted, was reported to act synergistically with glucocorticoids in autoimmune disease and even to be effective in patients insensitive to glucocorticoid treatment alone [37].

Endothelial activation by cytokines plays a fundamental role in the inflammation process. Here, we showed for the first time that tripterine, at nanomolar doses, could strongly inhibit the expression of ECAM triggered by different proinflammatory cytokines in human endothelium. Tripterine also reduced adhesion of leukocytes, especially monocytes and T lymphocytes, to activated human endothelium, casting a new light on its documented, in vivo, anti-inflammatory effects [15, 16, 18, 19]. In conclusion, based on the effects reported here, tripterine conjugated with its antioxidant and anticytokine effects, could affect the inflammatory process at multiple steps, thus deserving further investigation in the perspective of developing a potential new, anti-inflammatory drug.

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