Eupatilin Inhibits T-Cell Activation by Modulation of Intracellular Calcium Flux and NF-κB and NF-AT Activity

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ABSTRACT

Eupatilin, one of the pharmacologically active ingredients of *Artemisia princeps*, exhibits a potent anti-ulcer activity, but its effects on T-cell immunity have not been investigated. Here, we show that eupatilin has a profound inhibitory effect on IL-2 production in Jurkat T cells as well as in human peripheral blood leukocytes. Eupatilin neither influenced clustering of CD3 and LFA-1 to the immunological synapse nor inhibited conjugate formation between T cells and B cells in the presence or absence of superantigen (SEE). Eupatilin also failed to inhibit T-cell receptor (TCR) internalization, thereby, suggesting that eupatilin does not interfere with TCR-mediated signals on the membrane proximal region. In unstimulated T cells, eupatilin significantly induced apoptotic cell death, as evidenced by an increased population of annexin V^+/PI^+ cells and cleavage of caspase-3 and PARP. To our surprise, however, once cells were activated, eupatilin had little effect on apoptosis, and instead slightly protected cells from activation-induced cell death, suggesting that apoptosis also is not a mechanism for eupatilin-induced T-cell suppression. On the contrary, eupatilin dramatically inhibited I-κBα degradation and NF-AT dephosphorylation and, consequently, inhibited NF-κB and NF-AT promoter activities in PMA/A23187-stimulated T cells. Interestingly, intracellular calcium flux was significantly perturbed in cells pre-treated with eupatilin, suggesting that calcium-dependent cascades might be targets for eupatilin action. Collectively, our results provide evidence for dual regulatory functions of eupatilin: (1) a pro-apoptotic effect on resting T cells and (2) an immunosuppressive effect on activated T cells, presumably through modulation of Ca^{2+} flux. J. Cell. Biochem. 108: 225–236, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: EUPATILIN; IMMUNOSUPPRESSION; IL-2; APOPTOSIS; T-CELL ACTIVATION; CALCIUM

INTRODUCTION

The herb *Artemisia princeps* is a traditional oriental medicine that has been used for the treatment of diseases such as inflammation, microbial infection, and cancer. Following such use, a novel antipeptic formulation prepared from ethanol extracts of *A. princeps*, namely, DA-9601 (Stillen[TM]), has been developed to treat patients with gastric mucosal ulcers and is now commercially available in South Korea and other Asian countries [Hahm et al., 1998; Ryu et al., 1998; Oh et al., 2001; Huh et al., 2003; Choi et al., 2006]. DA-9601 has been reported to possess anti-oxidative and anti-inflammatory activities on experimentally induced gastrointestinal damage as well as hepatic and pancreatic lesions [Hahm et al., 1998; Ryu et al., 1998; Oh et al., 2001; Huh et al., 2003].

Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone) is an important ingredient of DA-9601 and has been reported to possess a variety of biological activities in vitro and in vivo. For example, eupatilin exhibits a cytoprotective function in some cells. Eupatilin protects smooth muscle cells from indomethacin-induced cell damage [Song et al., 2008] and gastric epithelial cells from oxidative damage [Choi et al., 2008; Lee et al., 2008]. Eupatilin also attenuates bile acid-induced hepatocyte apoptosis [Mine, 2006; Park et al., 2006]. In contrast, eupatilin exhibits a pro-apoptotic function in other cell types. Eupatilin inhibits the growth of MCF-10A-*ras* cells

and induces apoptotic cell death in human pro-myelocytic leukemia cells [Kim et al., 2005a,b; Seo and Surh, 2001]. In addition to its cytoprotective or pro-apoptotic functions, other functions have also been reported. Eupatilin enhances hepatic glucose metabolism and pancreatic beta-cell function in murine models of type 2 diabetes [Kang et al., 2008], and has anti-allergy [Lee et al., 2007] and anti-ulcer effects [Kim et al., 1997]. Interestingly, however, no previous studies have reported whether eupatilin directly exhibits immunosuppressive activity. In the present study, we focused on T lymphocytes and investigated the immunosuppressive effect of eupatilin on T cells in vitro, exploring its potential mode of action.

T cells play a pivotal role in immune responses. Activated T cells have been implicated in the pathogenesis of a variety of autoimmune disorders, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [Xiao and Link, 1999; VanderBorght et al., 2001]. Therefore, controlling the activation of pathogenic T cells is an important strategy for developing therapies and therapeutics against autoimmune disorders.

T-cell activation usually occurs by the engagement of T-cell receptors (TCRs) upon interaction with antigen-presenting cells (APCs) [Qian and Weiss, 1997]. Upon triggering by specific antigens (Ag), the TCR/CD3 complex initiates a complex series of biochemical processes inside the cell, culminating in various effector functions including cytokine production and the subsequent proliferation of T cells [Qian and Weiss, 1997]. One of the many events known to be important for the initiation of these processes is the activation of membrane proximal signals, including ZAP-70 and LAT, and the formation of an immunological synapse (IS) between T cells and APCs. The TCR/CD3 complex localizes to the center of the IS, or central supra-molecular activation center (cSMAC), whereas LFA-1 and adhesion molecules segregate to the peripheral region (pSMAC) [Monks et al., 1998]. Blocking of adhesion or TCR signaling inhibits conjugate formation, organization of IS, and IL-2 production [Ballard, 2001; Luo et al., 2007]. Another central event in T-cell activation is the pathway involving the nuclear factors of activated T cells. The transcription factors NF-κB and NF-AT play essential roles in the expression of IL-2, which promotes T-cell proliferation by interacting with the IL-2 receptor (IL-2R). Engagement of the TCR or phorbol 12-myristate 13-acetate (PMA)/Ca^{2+} ionophore activates the NF-κB and NF-AT pathways, resulting in the translocation of these transcription factors. Therefore, blocking the activity of these central transcription factors or their upstream second messengers, such as PKCs or Ca^{2+} and Ca^{2+}-dependent enzymes, significantly affects T-cell activation [Nelson and Willerford, 1998].

Apoptosis is another mechanism for maintaining cellular homeostasis both in physiologic and pathologic conditions [Krammer et al., 2007; Opferman, 2008]. Evidence has suggested that T-cell apoptosis is also actively involved in immunosuppression in various circumstances [Krammer et al., 2007; Opferman, 2008]. For example, apoptotic cells inhibit the expression of CD69 during T-cell activation [Sun et al., 2000; Sun and Shi, 2001]. Furthermore, it is well known that patients receiving cyclosporine, a potent immunosuppressant treatment, often develop autoimmunity upon cyclosporine withdrawal, which has been proposed to be due to the inhibition of T-cell apoptosis [Shi et al., 1989]. Therefore, controlling apoptosis of T cells will also provide novel strategies for the management of autoimmune diseases and transplantation [Krammer et al., 2007; Opferman, 2008].

In this study, we provide the first evidence of the effect of eupatilin on T-cell activation in vitro. Our results also show possible mechanisms of action of eupatilin on T-cell activation and elucidate the action of eupatilin on intracellular Ca^{2+} flux and two major transcription factors, NF-κB and NF-AT. We suggest that eupatilin may have therapeutic efficacy in a variety of immune disorders associated with over-activation of T cells.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES
Eupatilin was supplied by Dong-A Pharmaceutical Co. Ltd., (Yongin, South Korea) and dissolved in dimethyl sulfoxide (DMSO) for treatment. Unless otherwise indicated, 0.1% DMSO solution was used as a vehicle control. PMA, Ca^{2+} ionophore (A23187), cyclosporine A (CSA), and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Staphylococcus Enterotoxin E (SEE) was purchased from Toxin Technology (Sarasota, FL). Fluo-3 AM, CellTracker™ Green CMFDA (5-chloromethyfluorescein diacetate), and Orange CMRA were purchased from Molecular Probes (a division of Invitrogen, Eugene, OR). Phycoerythrin (PE)-conjugated AnnexinV and PE-conjugated mouse anti-human TCRαβ antibody were purchased from BD Biosciences (San Diego, CA). Antibodies to human LFA-1 (TS2/4) and CD3 (OKT3) were purified from hybridomas ATCC HB-202 and ATCC CRL-8001, respectively. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG was purchased from Sigma Chemical Co. Rabbit polyclonal antibodies against human cleaved caspase-3 (cleaved at Asp175), P53 (Do-1), cleaved PARP (cleaved at Asp214), and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Goat polyclonal anti-human Lamin B (C-20) antibody was purchased from Santa Cruz Biotechnology, Inc (Santa cruz, CA). Mouse anti-human NFAT-1 antibody that specifically detects both phosphorylated and dephosphorylated forms of NF-AT was purchased from BD Transduction Laboratories (San Jose, CA). Mouse monoclonal anti-human CD28 antibody was purchased from R&D Systems, Inc. (Minneapolis, MN).

CELL CULTURE
Jurkat T cells (ATCC CRL-1651, Manassas, VA), Raji B cells (kindly provided by Dr. Andrés Alcover, Pasteur Institute, Paris, France), and human peripheral blood leukocytes (PBLs) were grown in RPMI medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). All cell lines and human PBLs mentioned above were cultured at 37°C in a humidified incubator containing 5% CO_2 and 95% air.

MEASUREMENT OF IL-2 PRODUCTION
Jurkat T cells (5×10^5) or human PBLs (3×10^6) with or without eupatilin treatment were stimulated for the indicated times in the text with various stimuli including anti-CD3/CD28 antibodies, SEE-loaded Raji B cells, or PMA/A23187. For the anti-CD3/CD28 stimulation, cells were added on the culture dish coated with anti-CD3 (OKT3) antibody (10 μg/ml). Anti-CD28 antibody (10 μg/ml)

was treated right after the addition of cells. Stimulation of T cells by SEE-loaded Raji B cells was conducted as described in the Flow Cytometry-Based Conjugate Assay Section. The supernatants were collected at the indicated time points. Concentrations of IL-2 in the supernatants were measured using the Duoset Human IL-2 ELISA kit (R&D Systems, Inc.) according to the manufacturer's instructions.

FLOW CYTOMETRY–BASED CONJUGATE ASSAY

Jurkat T cells and Raji B cells were stained with CellTracker Orange CMRA (Molecular Probes) and Green CMFDA according to the manufacturer's directions, respectively. Raji B cells (2×10^5) were incubated in the presence or absence of SEE ($5 \mu g/ml$) for 30 min, washed, and resuspended in RPMI medium. Jurkat T cells were pre-incubated for 30 min with or without eupatilin ($100 \mu M$) and washed in RPMI medium. For conjugation, equal numbers of B and T cells were mixed and incubated at $37^\circ C$ for 2 h. The relative proportion of orange, green, and orange–green events in each tube was determined by two-color flow cytometry with a Coulter EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA). The number of gated events counted per sample was at least 10,000. The percentage of conjugated T cells was determined as the number of dual-labeled (CMFDA and CMRA-positive) events divided by the number of CMRA positive T cells as previously described [Morgan et al., 2001].

IMMUNOFLUORESCENCE STAINING AND CONFOCAL IMAGING ANALYSIS

Jurkat T cells were incubated with or without eupatilin ($100 \mu M$) for 1 h. Raji B cells were incubated with $1 \mu M$ of CellTracker Orange CMRA for 30 min. After two washes and resuspension in RPMI, Raji B cells were loaded with SEE ($5 \mu g/ml$) for 30 min at $37^\circ C$ in a humidified incubator containing 5% CO_2 and 95% air. SEE-loaded Raji B cells or unloaded Raji B cells were incubated with eupatilin-treated or -untreated Jurkat T cells on glass coverslips (18-mm diameter; Fisher Scientific, Pittsburgh, PA). After incubation for 1 h, the cells were fixed with 3.7% formaldehyde in PBS and washed twice with PBS. The cells were then incubated with anti-LFA-1 (TS2/4) or anti-CD3 antibodies in blocking buffer overnight at $4^\circ C$, rinsed three times with PBS, incubated with FITC-conjugated goat anti-mouse (Molecular Probes) antibody in blocking buffer for 1 h at room temperature, rinsed three times with PBS, and mounted with anti-fade solution (Molecular Probes). The slides were examined with a FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with $100 \times$ objectives.

TCR INTERNALIZATION ASSAY

TCR internalization was examined by flow cytometry as described previously [Davanture et al., 2005]. Briefly, Jurkat T cells (1×10^6) were pre-incubated in the absence or presence of eupatilin ($100 \mu M$) for 30 min, and each sample was incubated with PMA (200 nM) at $37^\circ C$ in a humidified incubator containing 5% CO_2 and 95% air. At the indicated time points, samples were divided and labeled with PE-conjugated anti-TCR$\alpha\beta$ mAb (Molecular Probes) at $4^\circ C$ for 1 h. After two washes in cold PBS, fluorescence of remaining surface TCR on the membrane was measured with a Coulter EPICS XL flow cytometer (Beckman Coulter).

MTT ASSAY

Cell viability was examined by MTT assay as described previously [Ben Trivedi et al., 1990]. A stock solution of MTT was prepared in PBS, diluted in RPMI1640 medium, and added to cell-containing wells at a concentration of 0.5 mg/ml after removing the culture medium. The plates were then incubated for 4 h at $37^\circ C$ in 5% CO_2. At the end of the incubation, the medium was aspirated, and the formazan product was solubilized with DMSO. Absorbency was measured on a VersaMax multiplate reader (Molecular Devices, CA) with a 570 nm wavelength filter. All experiments were performed at least three times.

APOPTOSIS

Apoptosis-mediated cell death of Jurkat T cells and human PBLs were examined using a double staining method with PE-labeled Annexin V/PI. A working solution of PE-annexin V was made from stock PE-annexin V ($0.1 \mu g/\mu l$) diluted 1:3,000 in Hanks' balanced salt solution (HBSS) supplemented with 2.5 mM $CaCl_2$. Jurkat T cells (1×10^6) and human PBLs (1×10^6) were added to $200 \mu l$ of the working solution of PE-annexin V and incubated for 10 min at $37^\circ C$. Then, the cells were further incubated with an equal volume of HBSS containing PI ($20 \mu g/ml$) and immediately analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter). All experiments were performed at least three times unless otherwise indicated.

CELL CYCLE ANALYSIS

Jurkat T cells were treated with indicated concentrations of eupatilin for 16 h. The cells were collected, washed with cold PBS, and fixed with 70% ethanol for 30 min at $4^\circ C$. The fixed cells were then washed with PBS and stained with $20 \mu g/ml$ of PI containing $10 \mu g/ml$ RNase A in the dark at room temperature for 20 min. The DNA content of the cells (1×10^5 cells per group) was analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter).

DAPI STAINING

Jurkat T cells were treated with or without eupatilin ($100 \mu M$) and fixed in 2% paraformaldehyde for 30 min at room temperature. The cells were subsequently pelleted onto a glass slide using a cytospin centrifuge at 500 rpm. The slide was incubated for 10 min in PBS containing DAPI (500 ng/ml) at room temperature, and the cells were examined under a fluorescence microscope equipped with a DAPI filter.

WESTERN BLOTTING

Cells were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM 4-nitrophenyl phosphate, $10 \mu g/ml$ leupeptin, $10 \mu g/ml$ pepstatin A, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) for 1 h on ice and centrifuged at 20,200 at $4^\circ C$ for 20 min. The protein samples were separated through 12% SDS–PAGE gels and transferred onto a nylon membrane by means of a Trans-Blot SD semidry transfer cell (Bio-Rad, Hercules, CA). The membrane was blocked in 5% skim milk (1 h), rinsed, and incubated with the indicated antibodies in TBS containing 0.1% Tween-20 (TBS-T) and 3% skim milk overnight. Excess primary antibody was removed by

washing the membrane four times in TBS-T. The membrane was then incubated with 0.1 µg/ml peroxidase-labeled secondary antibody (against rabbit or mouse) for 2 h. After three washes in TBS-T, bands were visualized by ECL Western Blotting detection WEST-20L plus (iNtRON Biotechnology, Inc., Korea).

MEASUREMENT OF CALCIUM INFLUX

Calcium influx was analyzed on a FV1000 confocal laser scanning microscope (Olympus) as previously described [Feske et al., 2001]. Jurkat T cells (2×10^6) were loaded with 1 µM Fluo-3 AM (Molecular Probes) in loading medium (RPMI + 10% FBS) for 1 h at room temperature, washed, and resuspended in loading medium. During the incubation period, each sample was tapped at 10 min intervals. The stained cells were attached onto PLL-coated coverslips for 15 min and mounted in a PC-R-10 bath flow chamber (Live Cell Instruments, Seoul, Korea). Unless otherwise indicated, cells were stabilized in a flow chamber for 10 min. After stimulation of cells with the various reagents, fluorescence intensity was detected at 488 nm and calculated for each 10-s interval after subtraction of background. For each experiment, 100–200 cells were analyzed. In experimental samples, cells were pre-treated with eupatilin (100 µM) or EGTA (2 mM) for 10 min before stimulation with anti-CD3 (OKT3) antibody or calcium activators.

LUCIFERASE ASSAYS

Jurkat T cells (1.5×10^6) were mixed with 3 µg of pGL3-NF-κB and NF-AT Luc plasmids with pRL-TK in 100 µl of nucleofector in a 4-mm cuvette. The cells were then transfected by Amaxa, Nucleofector[TM] system (Amaxa, Germany), transferred to 6-well plates containing 2 ml of complete growth medium, and allowed to recover for 48 h. Transfected cells were then treated with various agents as indicated in the text or figure legends. After 12 h of incubation, the cells were harvested and lysed in a lysis buffer (Promega, Madison, WI). Proteins were extracted by a freeze–thaw cycle, and cellular debris was removed by centrifugation at 4°C for 20 min. Luciferase activity was measured with a Centro LB 960 Luminometer (Berthold Technologies, Germany) according to the manufacturer's instructions. Reporter activity was presented as fold induction of luciferase activity over that of control cells.

STATISTICS

The mean values were calculated from data taken from at least three (usually three or more) separate experiments conducted on separate days. Where significance testing was performed, an unpaired Student's t-test and one-way ANOVA test were used. We considered differences between groups significant at $P < 0.05$.

RESULTS

EUPATILIN INHIBITS IL-2 SECRETION IN JURKAT T CELL

IL-2 is produced and released upon T-cell activation. Therefore, we examined whether eupatilin inhibits IL-2 production in activated Jurkat T cells. Treatment of Jurkat T cells with PMA (200 nM) in combination with calcium ionophore A23187 (1 µM) strongly stimulated IL-2 production with the levels increasing rapidly as the incubation time increased to 24 h. In contrast, pre-treatment with

eupatilin (100 µM) dramatically inhibited PMA/A23187-induced IL-2 production in Jurkat T cells. The results from 12 h cultures showed that eupatilin dose-dependently inhibited IL-2 production and that maximum inhibition was obtained at a concentration of 100 µM (Fig. 1A, right). Eupatilin also strongly inhibited anti-CD3/CD28 antibody-induced IL-2 production in a time-dependent manner (Fig. 1B). To mimic a physiologic response, Jurkat T cells were allowed to form conjugates with superantigen (SEE)-pulsed Raji B cells. IL-2 production was then analyzed at the indicated time points in the presence or absence of eupatilin. As shown in Figure, pre-treatment with eupatilin (100 µM) significantly inhibited IL-2 production in Jurkat T cells with SEE-pulsed Raji B cells. More supportively, pretreatment of human PBLs with eupatilin also significantly reduced IL-2 production induced by PMA/A23187 (Fig. 1D). Taken together, these results demonstrate that eupatilin has a profound inhibitory effect on T-cell function.

EUPATILIN DOES NOT BLOCK IMMUNOLOGICAL SYNAPSE FORMATION AND TCR INTERNALIZATION

T-cell activation requires sustained TCR interaction with major histocompatibility complex (MHC)-peptide complexes in the IS of the T cell–APC contact [Norcross, 1984; Paul and Seder, 1994]. We therefore performed a conjugation assay after Jurkat T cells were incubated with SEE-pulsed or un-pulsed Raji B cells. As shown in Figure 2A, pre-treatment with eupatilin showed little effect on SEE-induced conjugate formation between Jurkat T cells and Raji B cells. The mature IS was also examined by measuring clustering of membrane proteins, including TCR (cSMAC) and LFA-1 (pSMAC). However, clustering of CD3 and LFA-1 in the SMAC was not inhibited in the presence of eupatilin (Fig. 2B). Since activation of T cells also leads to the internalization of TCR, we also tested the effect of eupatilin on TCR internalization. As shown in Figure 2C, PMA-induced TCR internalization was also not blocked by eupatilin. These results demonstrate that eupatilin does not block the initial membrane events for T-cell activation.

EUPATILIN INDUCES APOPTOSIS IN RESTING UNSTIMULATED JURKAT T CELLS BUT NOT IN ACTIVATED JURKAT T CELLS

Apoptosis also can be an important mechanism for the inhibition of T-cell activation. Since eupatilin has been known to arrest cell cycle progression and induces apoptotic cell death in various cell types [Seo and Surh, 2001; Kim et al., 2004, 2005b; Mine, 2006], we further asked whether eupatilin induces cell cycle arrest or apoptosis in Jurkat T cells. As presented in Figure 3A, eupatilin did not significantly induce cell cycle arrest at a specific phase. However, the percentage of cells in the sub-G_1 phase, an indicator of apoptosis, was significantly elevated with increasing eupatilin concentration. Cell viability was assessed by MTT assay and revealed that eupatilin significantly reduced cell viability in a dose-dependent manner (Fig. 3B). In accordance with reduced cell viability, there was an increased proportion of annexin V^+/PI^+ cells after 16 h of incubation with various concentrations of eupatilin (Fig. 3C). To further verify the eupatilin-induced apoptotic cell death, the levels of active-caspase-3, cleaved PARP, and Lamin B were determined by Western blotting. As shown in Figure 3D, Lamin B was absent at the time that active-caspase-3 was detectable.
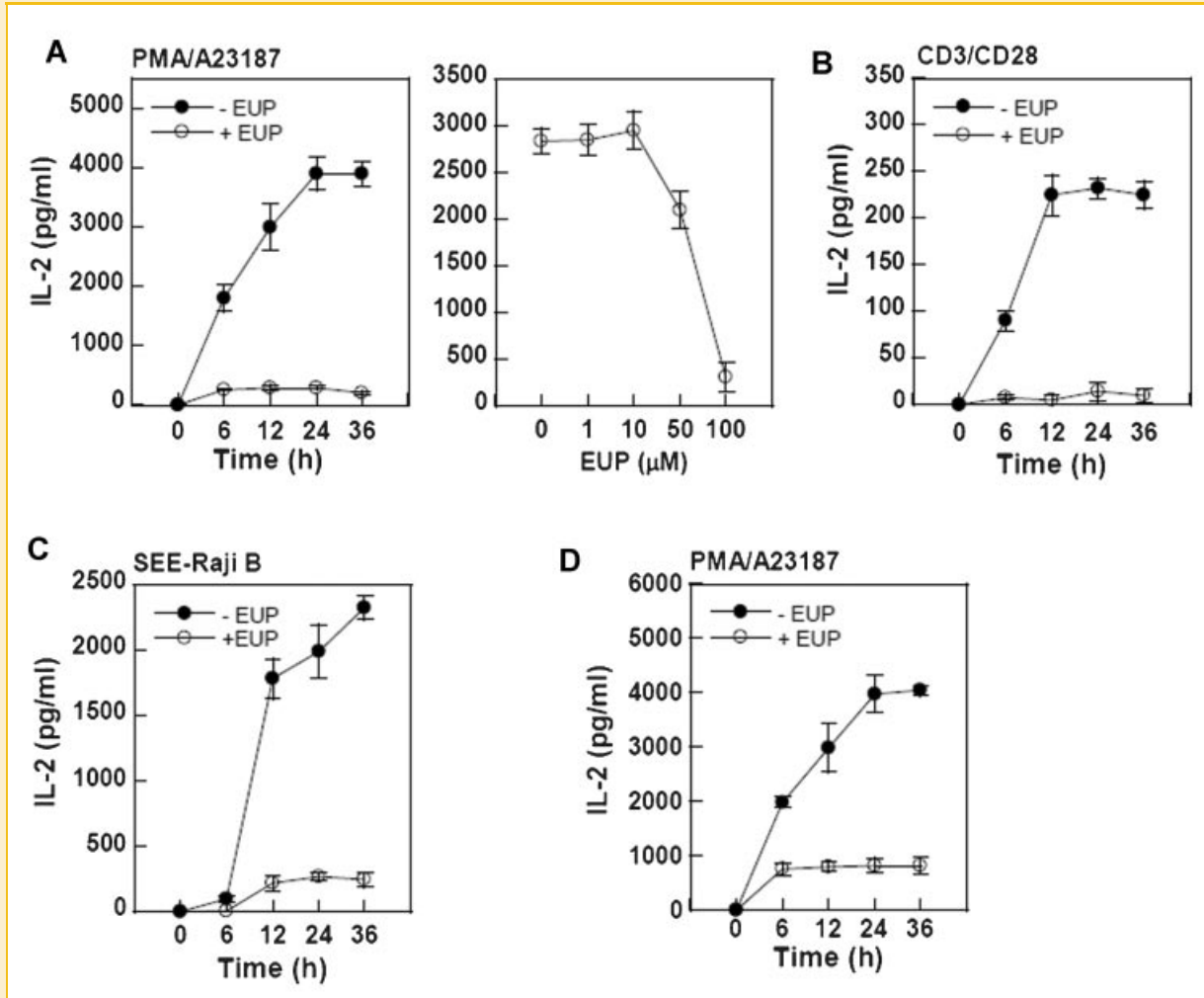
Fig. 1. Eupatilin inhibits IL-2 production in activated T cells. A–C: Jurkat T cells (5×10^5/well) were pre-incubated with or without eupatilin (100 μM) and stimulated with PMA (200 nM)/A23187 (1 μM) (A, left), anti-CD3/CD28 antibodies (10 μg/ml for each) (B), or co-cultured with SEE (5 μg/ml)-loaded Raji B cells (C). D: Human PBLs (3×10^6) were pre-incubated with or without eupatilin (100 μM) and then stimulated with PMA/A23187. At the indicated time points, the supernatants were collected and IL-2 secretion was measured by ELISA. (A, right) Jurkat T cells were also pre-incubated with various concentrations of eupatilin (0–100 μM). After 16 h of incubation, IL-2 was measured as described above. Results are expressed as mean ± SD of three independent experiments.

Cleaved PARP was also detected after 12 h of incubation with eupatilin (Fig. 3D).

Having determined that eupatilin induces apoptosis in resting Jurkat T cells, we next examined whether eupatilin also induces apoptosis in activated Jurkat T cells and whether the degree of apoptosis correlates with the inhibition of IL-2 production. To this end, cells were pre-treated with eupatilin for 30 min, and the cells were further incubated for 16 h with or without PMA/A23187. Flow cytometric analysis revealed that over 30% and 19% of cells were annexin V$^+$/PI$^+$ in the eupatilin- and PMA/A2318-treated groups, respectively (Fig. 4A). Surprisingly, however, combination treatment with eupatilin and PMA/A23187 significantly reduced the percentage of annexin V$^+$/PI$^+$ cells (Fig. 4A). This phenomenon was also similarly reproduced in human PBLs except the cell death by eupatilin alone in resting state in which cell death of human PBLs was slightly lower than Jurkat T cells (Fig. 4B). Treatment with eupatilin also blocked PMA/A23187-induced PARP cleavage as well

as nuclear fragmentation (Fig. 4C,D) in Jurkat T cells. Taken together, these results strongly demonstrate that apoptosis is not a mechanism for eupatilin-mediated inhibition of IL-2 production in activated T cells, though how eupatilin inhibits apoptosis in activated Jurkat T cells is not currently understood.

EUPATILIN INHIBITS TRANSCRIPTIONAL ACTIVITIES OF NF-κB AND NF-AT

A central event in T-cell activation is the pathway involving the nuclear factors of activated T cells. As shown in Figure 5A, eupatilin did not block PMA-induced ERK phosphorylation. Since the ERK pathway plays a critical role in IL-2 production and lies upstream of transcription factors such as the AP-1 family in T cells [Koike et al., 2003; Kim et al., 2007], this result may suggest that eupatilin inhibits IL-2 production through blockade of an ERK-AP-1-independent pathway. We therefore tested whether eupatilin inhibits the transcriptional activities of two other major transcription factors,
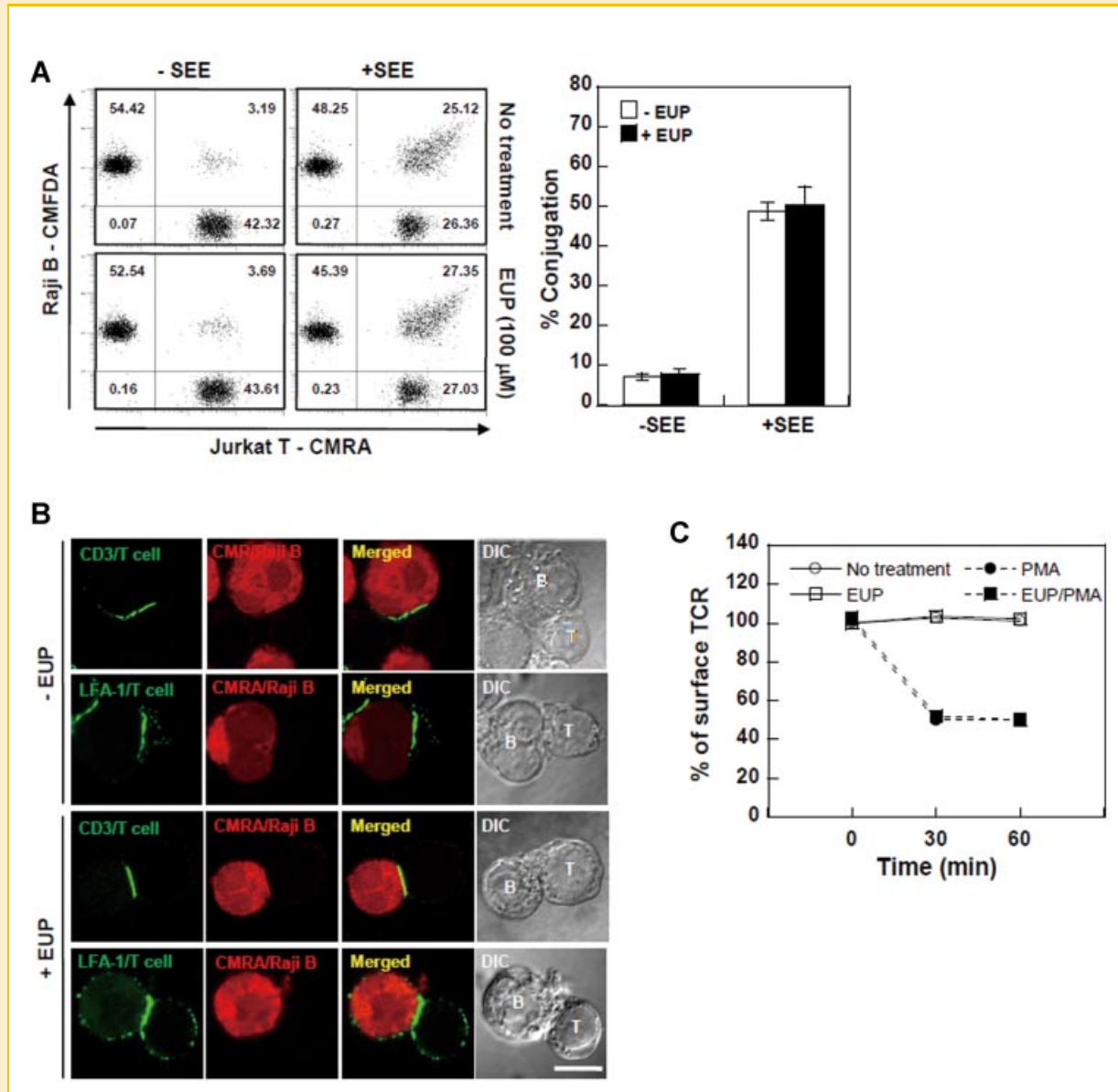
Fig. 2. Eupatilin does not affect antigen-dependent conjugate formation. A: Jurkat T cells and Raji B cells were stained with CMFDA and CMRA, respectively. Raji B cells (2×10^5) were pre-incubated with or without SEE (5 μg/ml) and mixed with the same number of untreated or eupatilin-treated Jurkat T cells. Conjugate formation was analyzed by flow cytometry (left), and the data are represented as the percentage of conjugated cells (right) as described in the Materials and Methods Section. B: The conjugated cells from above (A) were fixed and stained with anti-CD3 and anti-LFA-1 antibodies. These data are representative of three independent experiments. The images were taken by confocal microscopy. Scale bars = 10 μm. C: Jurkat T cells (5×10^5) were pre-treated with or without eupatilin and were then incubated with PMA for various time periods. TCRs on the surface of T cells were detected with anti-TCRαβ antibody by flow cytometry. Results are expressed as mean ± SD of three independent experiments.

NF-κB and NF-AT. To this end, Jurkat T cells were transiently transfected with NF-κB or NF-AT reporter vectors, and the effect of eupatilin was then evaluated. As shown in Figure 5B,C, pretreatment with eupatilin significantly suppressed PMA/A23187-induced luciferase activities of both NF-κB and NF-AT. In keeping with these results, treatment with eupatilin blocked PMA/A23187-induced I-κBα degradation and NF-AT1 dephosphorylation events (Fig. 5D,E). Because of the excessive cell deaths, the cells which were treated with eupatilin alone or eupatilin plus A23187 showed lower

luciferase activities than the untreated control (Fig. 5B,C). Taken together, these results suggest that eupatilin inhibits IL-2 production in Jurkat T cells, at least in part, due to the blockade of the transcriptional activities of both NF-κB and NF-AT1.

EUPATILIN MODULATES INTRACELLULAR CALCIUM FLUX DISTINCTIVELY FROM EGTA

TCR triggering by the MHC-Ag peptide complex induces Ca^{2+} entry through plasma membrane Ca^{2+} channels, an indispensable step by
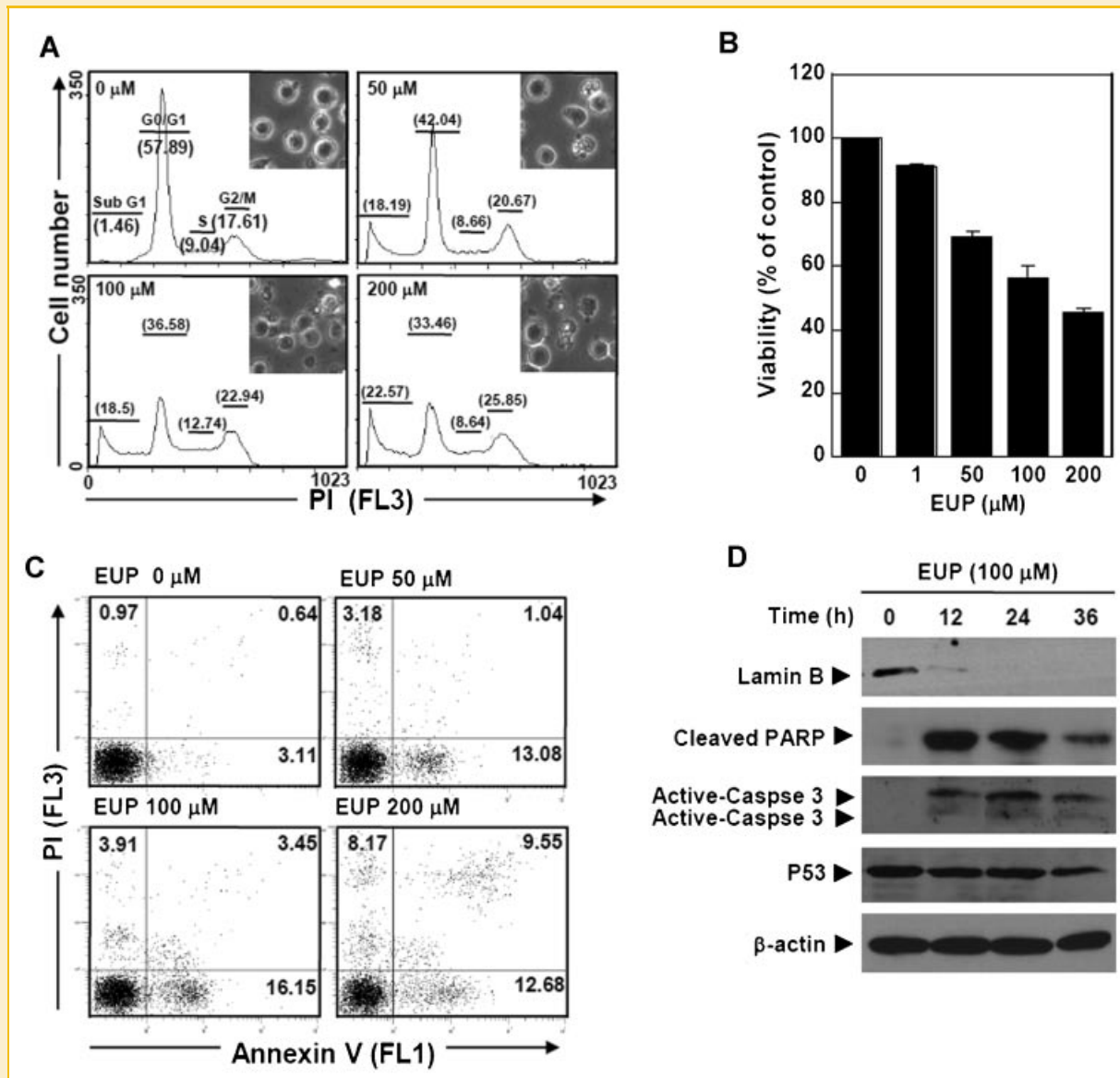
Fig. 3. Eupatilin induces cell death in resting T cells. A–C: Jurkat T cells (1×10^6) were treated with the indicated concentrations of eupatilin for various time periods. After 16 h of incubation, the cells were stained with PI, and the cell cycle was analyzed by flow cytometry (A). Note: estimated values of each cell cycle phase were indicated in the parenthesis. Cell viability was determined by MTT assay (B). The population of Annexin V$^+$/PI$^+$ cells was measured by flow cytometry (C). D: Jurkat T cells (2×10^6) were incubated with eupatilin (100 μM) for the indicated time points, and Western blotting was performed using antibodies against Lamin B, cleaved PARP, active–caspase3, and P53.

which T cells expand clonally and acquire effector functions. With the knowledge that activation of both NF-κB and NF-AT requires calcium signaling and, especially since dephosphorylation of NF-AT inevitably requires Ca^{2+}-calmodulin-dependent Ser/Thr protein phosphatase calcineurin activity [Bueno et al., 2002], we next questioned whether eupatilin modulates intracellular calcium flux in Jurkat T cells. To this end, Jurkat T cells were pre-stained with the Ca^{2+}-sensitive dye fluo-3 AM and treated with eupatilin, anti-CD3 Ab, A23187, eupatilin plus anti-CD3 Ab, or eupatilin plus A23187. In the case of simultaneous treatment, cells were treated with eupatilin for 10 min before anti-CD3 Ab or A23187, unless otherwise indicated. To our surprise, treatment with eupatilin not

only decreased intracellular Ca^{2+} content but also blocked anti-CD3 Ab- or A23187-induced Ca^{2+} influx during the early time periods (0–500 s) (Fig. 6A). These results suggest that eupatilin may directly or indirectly modulate intracellular Ca^{2+} influx. However, to determine whether eupatilin acts as a Ca^{2+} chelator like EGTA, we compared the modes of Ca^{2+} influx after eupatilin or EGTA treatment. As shown in Figure 6B, EGTA-mediated decrease of intracellular Ca^{2+} content was prolonged during the recording periods (0–2,000 s). In contrast, the eupatilin-mediated decrease of intracellular Ca^{2+} content gradually recovered to basal levels in the latter time periods (1,500–2,000 s). We next compared the effects of eupatilin and EGTA in terms of their inhibitory mode of action on
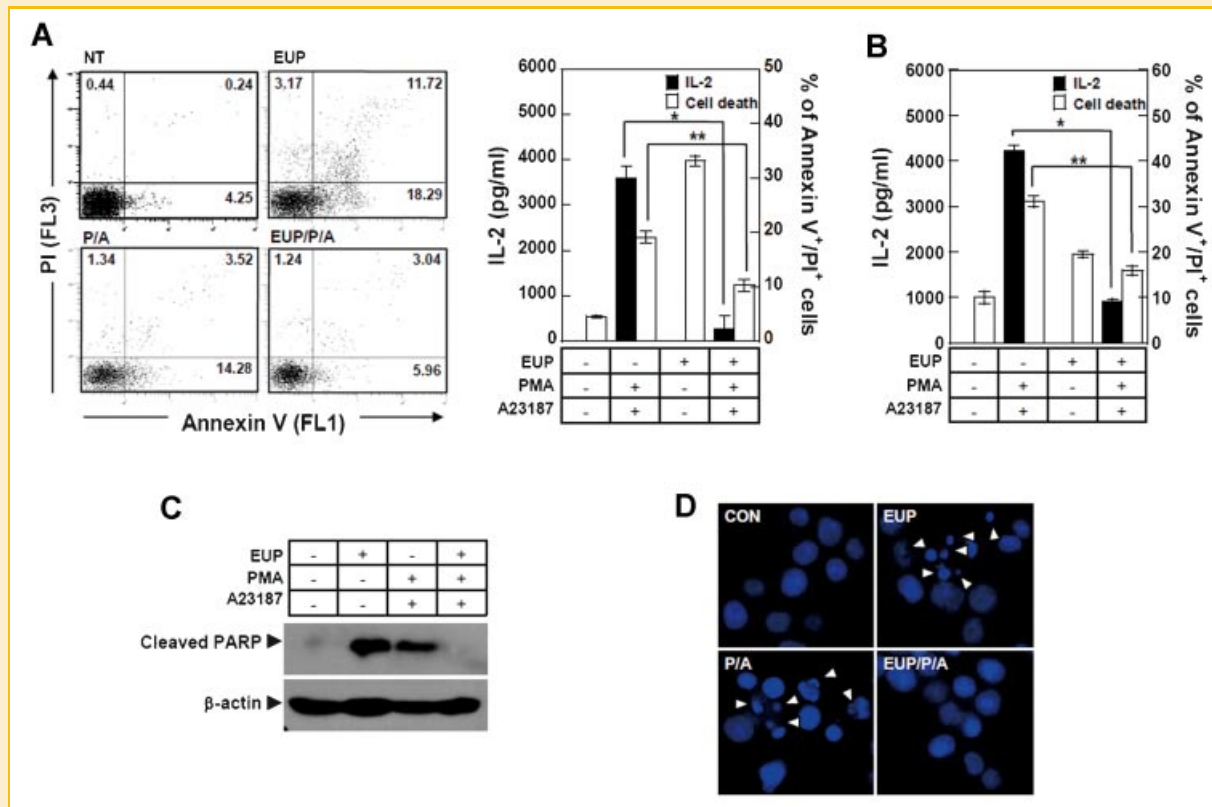
Fig. 4. Eupatilin does not induce apoptosis in activated T cells. Jurkat T cells (1×10^6) (A) and human PBLs (3×10^6) (B) were pre-treated with eupatilin (100 µM) for 30 min, and cells were subsequently stimulated with PMA (200 nM)/A23187 (1 µM) for 16 h. The degree of cell death (Annexin V and PI staining) was measured by flow cytometry and IL-2 production was measured by ELISA. Cell death and IL-2 expression were significantly suppressed by eupatilin. $^*P < 0.003$ (A), $^{**}P < 0.03$ (A), $^*P < 0.01$ (B), and $^{**}P < 0.001$ (B) versus PMA/A23187. PARP cleavage was determined by Western blotting (C). Apoptotic cells were also determined by staining with DAPI (500 ng/ml) (D). Note: apoptotic cells were identified based on the presence of granular nuclear apoptotic bodies (white arrowhead).

anti-CD3 Ab-induced Ca^{2+} influx. With longer time measurement, interestingly, we found that eupatilin not only slightly blocks but also significantly retards anti-CD3 Ab-induced Ca^{2+} influx (Fig. 6B, left). In contrast, this retardation was not seen in EGTA (2 mM)-treated Jurkat T cells (Fig. 6B, left). Treatment with EGTA completely blocked Ca^{2+} influx, but allowed the release of calcium from intracellular stores. We therefore determined the effect of eupatilin on thapsigargin, a selective inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases, -induced elevation of intracellular calcium and compared its mode of action with EGTA. As shown in Figure 6B (right), in contrast to EGTA, only eupatilin retarded thapsigargin-induced Ca^{2+} influx in Jurkat T cells. These results demonstrate that eupatilin does not act as a calcium chelator but may function as a calcium modulator, though how eupatilin modulates Ca^{2+} influx is not currently understood.

DISCUSSION

Previous studies suggested that eupatilin possesses a variety of biological activities in vitro and in vivo, and some of those activities may be mediated by the inhibition of NF-κB activity [Kim et al., 2007; Lee et al., 2007, 2008]. However, limited research has been carried out in relation to other potential bioactive properties of eupatilin, such as immunosuppressive activity. T lymphocytes play a pivotal role in the pathogenesis of cell-mediated autoimmune diseases and chronic inflammatory disorders [Perkins, 1998]. Since IL-2 production in T cells is an index of immune power [Paul and Seder, 1994], here we investigated the effects of eupatilin on IL-2 production in both Jurkat T cells and human PBLs and explored its potential mechanism of action.

Activation of PKC and elevation of calcium/calcineurin pathways are involved in a variety of signaling responses including IL-2 production in T cells [Clapham, 1995; Werlen et al., 1998]. We used two different activation systems, and hence mechanisms, to activate cytokine production in Jurkat T cells. By including anti-CD3/CD28 antibodies, SEE-pulsed Raji B cells, and PMA/A23187, we examined both TCR-dependent and TCR-independent pathways for IL-2 transcription and production [Tanaka et al., 2005]. IL-2 suppression by eupatilin occurred in cells stimulated with all three stimulating protocols. This suggests that eupatilin-induced suppressive effects are not dependent on TCR activation. In accordance with this observation, eupatilin failed to inhibit not only SEE-induced conjugate formation between Jurkat T cells and Raji B cells but also activation-induced clustering of CD3 and LFA-1 in the SMAC. In contrast, we found that eupatilin dramatically inhibited
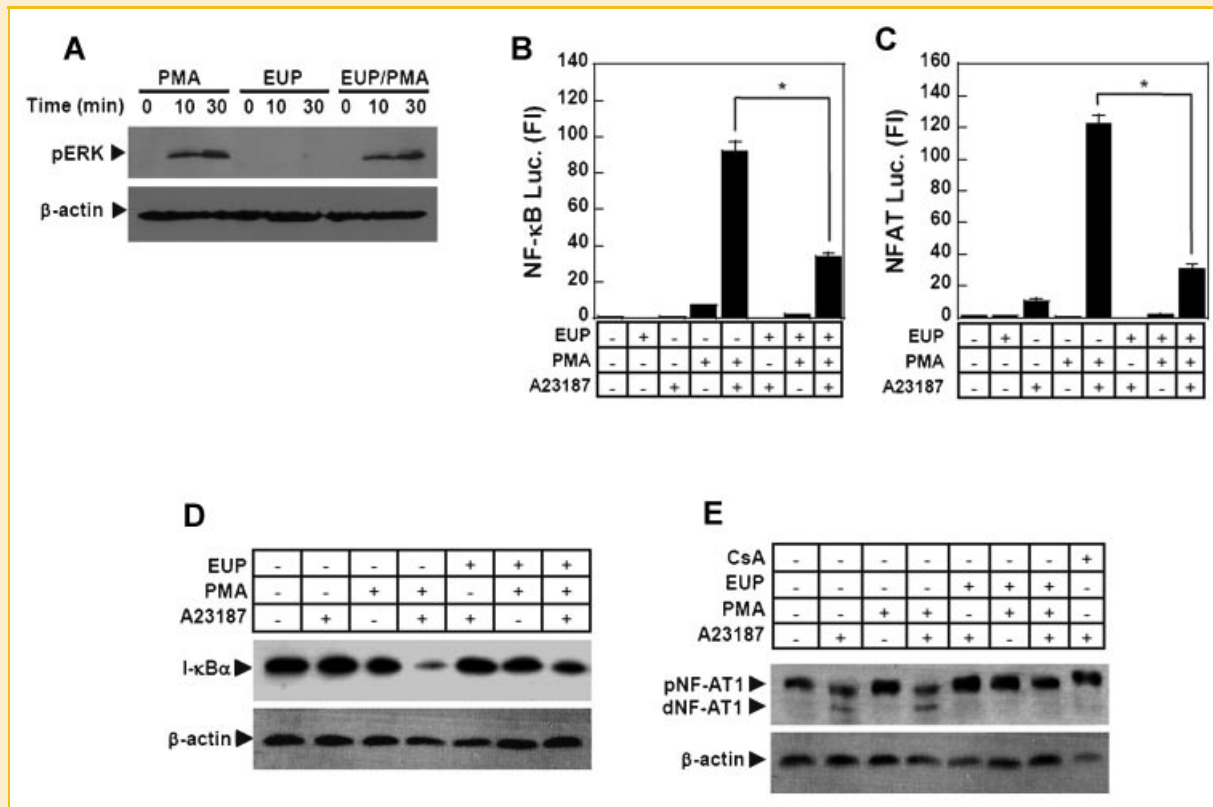
Fig. 5. Eupatilin inhibits NF-AT and NF-κB activation. (A) Jurkat T cells (1×10^6) were treated for the indicated time points with PMA (200 nM), eupatilin (100 μM), or PMA plus eupatilin. For the simultaneous treatment, cells were pre-treated with eupatilin for 30 min prior to PMA treatment. The cell lysates were analyzed for pERK by Western blotting. B,C: Jurkat T cells (1.5×10^6) were transfected with pGL3-NF-κB and NF-AT Luc plasmids with pRL-TK for 24 h. The cells were then treated with the indicated reagents (eupatilin, PMA, and A23187) for 16 h, and luciferase activities were measured by luminometer. $^*P < 0.005$ (B) and $^*P < 0.03$ (C) versus PMA/A23187. D,E: Jurkat T cells (1×10^6) were treated for 30 min with or without the indicated reagents [eupatilin, PMA, A23187, or CSA (1 μM)]. Cells were pre-treated with both eupatilin and CSA for 30 min before stimulation with other reagents. Western blotting was performed to determine the degradation of I-κBα and dephosphorylation of NFAT. Note: dNF-AT1, dephosphorylated form of NF-AT1; pNF-AT1, phosphorylated form of NF-AT1.

transcriptional activities of both NF-κB and NF-AT in Jurkat T cells. These findings strongly suggest that eupatilin blocks at the site or upstream of NF-κB and NF-AT in terms of IL-2 production in Jurkat T cells.

Notably, ERK phosphorylation was not inhibited by eupatilin. Since the ERK pathway lies upstream of transcription factor AP-1 in T cells [Koike et al., 2003; Tanaka et al., 2005], this result may suggest that eupatilin blocks IL-2 production via an ERK-AP-1-independent pathway. We therefore tested its potential effect on NF-AT phosphorylation status and found that eupatilin strongly inhibits NF-AT dephosphorylation. NF-AT proteins are known to be regulated by a reversible cycle of dephosphorylation and rephosphorylation. Increments of intracellular free Ca^{2+} levels trigger the activation of the Ca^{2+}-calmodulin-dependent Ser/Thr-specific phosphatase calcineurin, which is unique in its ability to dephosphorylate NF-AT proteins [Bueno et al., 2002]. Thus, inhibition of NF-AT dephosphorylation by eupatilin may suggest that eupatilin-mediated immunosuppressive effects are directly coupled with the ability of eupatilin to modulate intracellular Ca^{2+} levels.

Ca^{2+} is essential in lymphocyte activation and maturation. TCR engagement by MHC-Ag peptides results in sustained influx of extracellular Ca^{2+} across the plasma membrane and leads to consequent activation of many transcription factors, including NF-AT, NF-κB, and CREB [Dolmetsch et al., 1997]. Mutant T cells deficient for Ca^{2+} influx resulted in impaired IL-2 production and defective T cell-mediated immune responses [Feske, 2007]. In the current study, we found a distinctive pattern of Ca^{2+} influx in eupatilin-treated Jurkat T cells. This pattern was not observed in EGTA-treated T cells, suggesting that eupatilin does not act as a calcium chelator. In an agreement with this line, nuclear magnetic resonance (NMR) patterns of eupatilin with or without Ca^{2+} were not significantly different, thereby suggesting that a eupatilin-Ca^{2+} complex was not formed [Lee et al., 1997]. However, it is intriguing that eupatilin not only blocks but also significantly retards anti-CD3 Ab-induced Ca^{2+} influx. This result suggests a potential role for eupatilin in blocking Ca^{2+} channels that are critical for T-cell activation. In fact, the intracellular concentration of Ca^{2+} ions can be regulated through the sequential operation of two interdependent processes: depletion of endoplasmic reticulum Ca^{2+} stores as a result of binding of inositol trisphosphate (IP_3) to IP_3 receptors, followed by "store-operated" Ca^{2+} entry through plasma membrane Ca^{2+} channels [Oh-hora and Rao, 2008]. In lymphocytes, mast cells, and other immune cell types, store-operated Ca^{2+} entry through
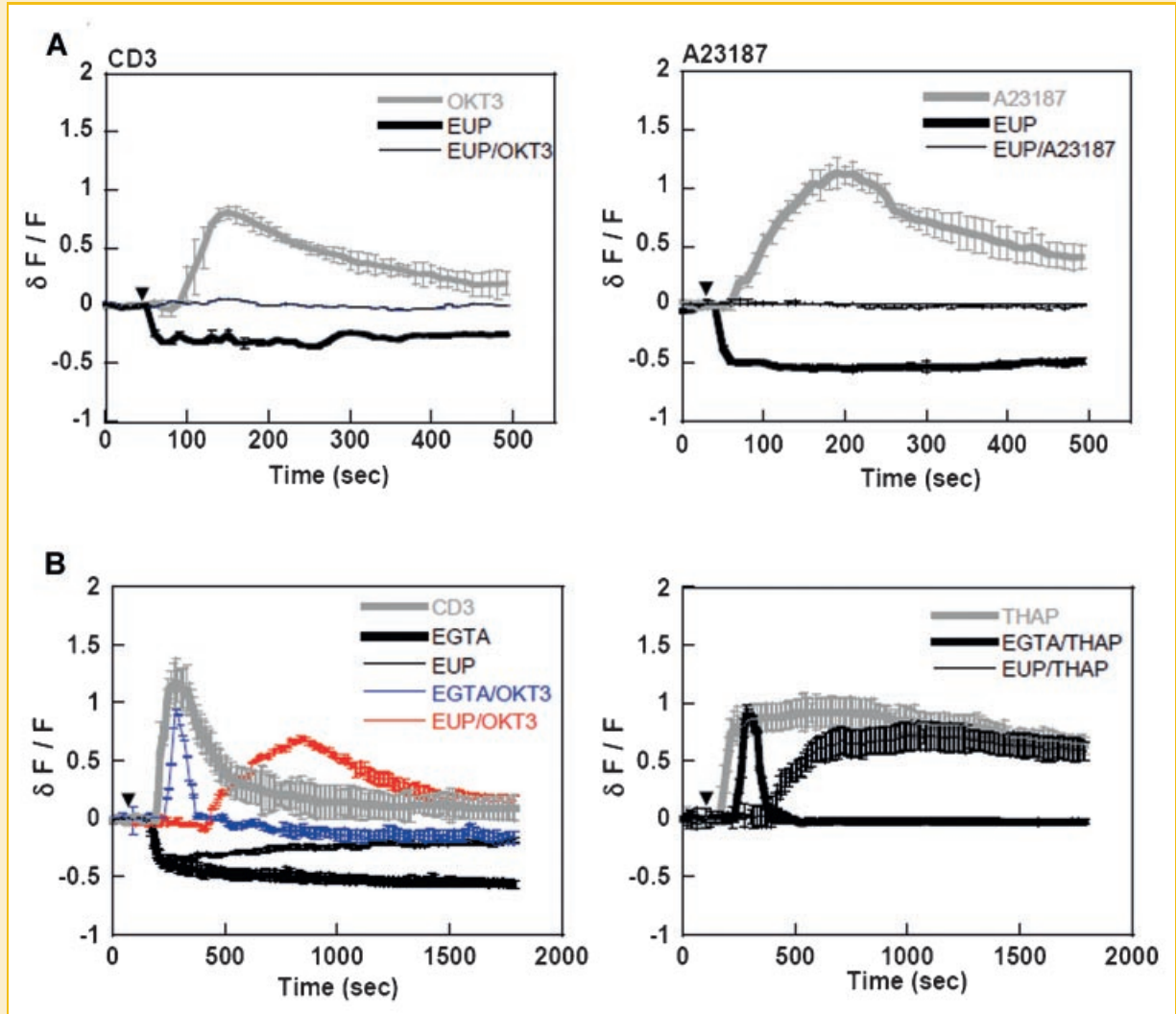
Fig. 6. Eupatilin modulates calcium influx. Jurkat T cells were loaded with Fluo–3 AM (1 μM) as described in Materials and Methods Section, and the cells were stimulated with various reagents (10 μg/ml of anti–CD3 mAb, 1 μM of A23187, 100 μM of eupatilin, 10 μM of thapsigargin, and/or 2 mM of EGTA) as indicated. For simultaneous treatment, cells were pre–treated with eupatilin or EGTA for 30 min before stimulation with other agents. Fluorescence was detected at 10–s intervals for 500 s (A) or 1,800 s (B) by confocal microscopy.

specialized Ca^{2+} release-activated calcium (CRAC) channels constitutes the major pathway of intracellular Ca^{2+} increase [Gwack et al., 2007; Oh-hora and Rao, 2008]. Therefore, it will be interesting to test the mechanism of action of eupatilin under stringent conditions, such as extracellular calcium depletion. Since calcium channel-regulating molecules, such as stromal interaction molecule (STIM) and ORAI, are also important for CRAC channel function, understanding whether eupatilin suppresses the function of these two essential regulators warrants further study.

Apoptosis is a conserved genetic program for the development and homeostasis of the immune system [Krammer et al., 2007; Opferman, 2008]. Although apoptosis participates in the development of virtually all cell lineages, it plays an essential role in the immune system, specifically by maintaining immune tolerance against self-antigens. In this respect, it was interesting to note that eupatilin significantly induced apoptotic cell death in unstimulated

Jurkat T cells. The mechanism by which eupatilin-induced apoptosis was similar to that demonstrated in previous studies in other cell types [Seo and Surh, 2001; Kim et al., 2004, 2005b]. For example, previous reports demonstrated that eupatilin-induced cell death is associated with the proteolytic cleavage of caspase-3 and PARP in HL-60 cells and AGS cells [Seo and Surh, 2001; Kim et al., 2004, 2005b], and we have reproduced these findings herein with Jurkat T cells. However, no effects on cell cycle arrest or p53 expression suggest that there is yet another unique apoptosis-inducing mechanism of eupatilin in Jurkat T cells.

It is well defined that although clonal expansion of T cells is needed to clear an antigen from the body, most of the cells need to be deleted after the antigenic stimulus is removed. Apoptosis is also an important mechanism in clearing antigen-reactive T cells and reducing their activation [Opferman, 2008]. In the present study, however, it was very striking to observe that eupatilin demonstrated

little effect on apoptosis, but rather slightly protected cells from activation-induced cell death (AICD). While the mechanism by which eupatilin blocks AICD in Jurkat T cells is not clear at present and also beyond of the scope of the current study, this result strongly suggests that apoptosis is not a mechanism for eupatilin-mediated suppression of T-cell activity. Further studies will be necessary to elucidate why eupatilin inhibits AICD in Jurkat T cells.

Taken together, the results of the current study reveal that eupatilin exhibits a novel immunosuppressive effect on T cells through the modulation of Ca^{2+} influx and subsequent blocking of NF-AT dephosphorylation. Apparently, these events followed the reduction of transcriptional activity of both NF-κB and NF-AT. These findings extend our understanding of the immunosuppressive effects of eupatilin. Although further investigation is needed, the current study suggests eupatilin as a potential new and effective treatment for the treatment of T-cell-mediated autoimmune diseases such as ulcerative colitis, Crohn's disease, and RA, especially in patients that require treatment with immunosuppressive agents.

ACKNOWLEDGMENTS

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