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Down-regulation of c-*jun* N-terminal kinase-activator protein-1 signaling pathway by *Ginkgo biloba* extract in human peripheral blood T cells

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Abstract

The activation of T lymphocytes contributes to inflammatory process of cardiovascular and cerebrovascular diseases. We investigated the effects of the extract of *Ginkgo biloba* (EGb), an ancient plant preserving antioxidant property, on phorbol 12-myristate 13-acetate + ionomycin or anti-CD3 + anti-CD28 monoclonal antibodies-activated T cells. Human peripheral blood T cells were negatively selected from whole blood. Cytokines were measured by ELISA, cell surface markers by flow cytometry and the activities of transcription factors and kinases were determined by electrophoresis mobility shift assays, kinase assays and transfection assays. We showed that EGb inhibited several cytokines, including tumor necrosis factor-alpha, interleukin (IL)-2, IL-4 and interferon-gamma production from activated T cells. Electrophoresis mobility shift assay analysis indicated that EGb down-regulated activator protein-1 (AP-1) but not nuclear factor kappa B DNA-binding activity. In addition, EGb inhibited c-*jun* N-terminal kinase but not extracellular signal regulated protein kinase activity. The inhibitory specificity on AP-1 by EGb was also demonstrated in transfection assays. The inhibition of AP-1 signaling pathway in T cells by EGb provides a support for its efficacy in cardiovascular and cerebrovascular diseases and raises a therapeutic potential for this drug in activated T cell-mediated pathologies.

Keywords: *Ginkgo biloba* extract; Cardiovascular diseases; T lymphocytes; Cytokines; c-*jun* N-terminal kinase; Activator protein-1

1. Introduction

Inflammation has been one of the major factors leading to the development of atherosclerosis, myocardial ischemia and cerebral ischemia [1–3]. In cardiovascular aspect, the extent of initial coronary plaque inflammation determines the rate of recurrence of unstable angina in patients receiving directional coronary atherectomy [4]. Moreover, inflammation, by itself, can affect plaque stability in restenotic coronary lesions that cause unstable coronary syndromes [5,6]. Inside the atherosclerotic lesions, the inflammatory infiltrates comprise mainly of activated macrophages and T lymphocytes [1,7,8]. These immune effector cells involved in inflammatory process in plaque lesions can also be reflected in the circulating populations [9,10]. Aside from the inflammatory cells, the cytokines secreted from these activated cells also play important roles in mediating the formation of atheroma, causing the instability of plaque as well as contributing to ischemia/reperfusion myocardial and cerebral injuries [11–17]. Through the secretion of a variety of cytokines, T cells play not only as initiators but also as regulators in inflammatory response of atherosclerotic lesions [18–21].

Ginkgo biloba, an ancient plant, its standardized extract of leaves has been extensively employed as a phytomedicine in

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Abbreviations: EGb, *Ginkgo biloba* extract; PMA, phorbol 12-myristate 13-acetate; IL, interleukin; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; NF-κB, nuclear factor kappa B; AP-1, activator protein-1; JNK, c-*jun* N-terminal kinase; ERK, extracellular signal regulated protein kinase; IL-2Rα, interleukin-2 receptor alpha chain; mAb, monoclonal antibody; EMSA, electrophoresis mobility shift assay.

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Europe and as a nutritional supplement in the United States [22]. The *Ginkgo biloba* extract (EGb) contains two active constituents preserving antioxidant properties, which include flavanoids (ginkgo-flavone glycosides) and terpenoids (ginkgolides and bilobalides). The commonly recognized therapeutic benefits for this drug in central nervous system diseases have been demonstrated in Alzheimer's disease, chronic refractory schizophrenia and in sleep disturbance of depressed patients [23–26]. Because of in part its antioxidant property, the therapeutic effects of EGb can also be observed in a variety of cardiovascular and cerebrovascular diseases [27–33]. Since T cells and T cell-derived cytokines play critical roles in inflammatory process of cardiovascular and cerebrovascular diseases, we were interested to know whether and how the EGb can possibly regulate the activation of T cells.

2. Materials and methods

2.1. Preparation of EGb and drug cytotoxicity measurement

The EGb powder, manufactured by Indena, was kindly provided by Yung Shin Pharmaceutical Ind. Co., Ltd. This is a standardized extract that contains two major active constituents flavanoids 24% and terpenoids 6%. The drug was dissolved in DMSO to make a stock concentration. For experiments, the required concentrations of EGb were made by further dilution of the concentrated stock solution with culture medium. The cell culture medium contained RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine and 1000 U/mL penicillin–streptomycin (Gibco-BRL). The final concentrations of DMSO were consistently 0.05% that showed no cytotoxicity to T cells. By trypan blue exclusion assays, LDH release assays and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assays analysis [34], we observed that EGb was not toxic when concentrations less than 100 μg/mL were used to treat human peripheral blood T cells (data not shown).

2.2. Preparation of human peripheral blood T cells

Human peripheral blood T cells were negatively selected from whole blood according to our previous report [35]. Briefly, buffy coat from blood bank was mixed with Ficoll-Hypaque, after centrifugation, the layer of mononuclear cells was collected. After lysis of red blood cells, the peripheral blood mononuclear cells were laid on Petri dishes to remove adherent cells and then incubated with antibodies, including L243 (anti-DR; American Type Culture Collection (ATCC)), OKM1 (anti-CD11b; ATCC), and LM2 (anti-Mac1; ATCC) for 30 min at 4°. The cells were then washed with medium containing 0.1% fetal bovine serum and incubated with magnetic beads conjugated with goat anti-mouse IgG (R&D). The antibody-stained cells were then removed with a magnet. Following a repeat of the above procedures, the T cells were obtained with purity more than 98% as determined by the percentage of CD3+ cells in flow cytometry (Beckton Dickinson).

2.3. Cell stimulation and cytokine ELISA

For cell activation, the following stimuli and concentrations were used: phorbol 12-myristate 13-acetate (PMA, Sigma) at 5 (for cytokine measurements and transfection assays) or 20 ng/mL (for the rest of the studies); ionomycin (Sigma) at 1 μM; immobilized anti-CD3 monoclonal antibodies (mAbs, OKT3, ATCC) at 10 μg/mL and soluble anti-CD28 mAbs (clone 9.3, kindly provided by Dr. Carl June, Naval Institute, NIH) at 1 μg/mL concentrations. The cells were incubated with a series of stimuli for variable time points and the cell pellets or supernatants were collected for further analysis. The determination of cytokine concentrations was performed as described in [34].

2.4. Measurement of cell surface molecule expression

Human peripheral blood T cells at a concentration of 5×10^5/mL were preincubated with various concentrations of EGb for 2 hr. After adding stimuli for another 24 hr, the cells were collected and washed with PBS. After the wash, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-IL-2 receptor alpha (anti-IL-2Rα or anti-CD25) or FITC-conjugated isotype-matched mAbs (PharMingen) and the expression of cell surface molecules was determined with a flow cytometer (Becton Dickinson). The percentages of each cell surface molecule expression were used to evaluate the drug effects.

2.5. Nuclear extract preparation

Nuclear extracts were prepared as described in [36]. Briefly, the treated cells were left at 4° in 70 μL of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM PMSF, and 3.3 μg/mL aprotinin) for 15 min with occasional gentle vortexing. The swollen cells were centrifuged at 25,000 *g*, 3 min after removal of the supernatants (cytoplasmic extracts), the pelleted nuclei was washed with 70 μL buffer A and subsequently, the cell pellets were resuspended in 30 μL buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 0.5 mM PMSF, and 3.3 μg/mL aprotinin) and incubated at 4° for 30 min with occasional vigorous vortexing. Then the mixtures were centrifuged at 25,000 *g*, 20 min and the supernatants were used as nuclear extracts.

2.6. Electrophoresis mobility shift assay (EMSA)

The EMSA was performed as described in [36]. The oligonucleotides containing nuclear factor kappa B

(NF-κB)-binding site (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) and AP-1-binding site (5′-CGC TTG ATG AGT CAG CCG GAA-3′) were purchased and used as DNA probes (Promega). The DNA probes were radiolabeled with [γ-^{32}P]ATP using the T4 kinase according to the manufacturer's instructions (Promega). For the binding reaction, the radiolabeled NF-κB or AP-1 probe was incubated with 5 μg of nuclear extracts. The binding buffer contained 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 5% glycerol, and 2 μg poly(dI-dC). The reaction mixture was left at room temperature to proceed binding reaction for 20 min. If unradiolabeled competitive oligonucleotides were added, they were used as 100-fold molar excess and preincubated with nuclear extracts for 10 min before the addition of the radiolabeled probes. The final reaction mixture was analyzed in a 6.6% nondenaturing polyacrylamide gel with 0.5X Tris–borate/EDTA (TBE) as an electrophoresis buffer.

2.7. Western blotting

ECL Western blotting (Amersham) was performed as described in [35]. Briefly, after extensive wash, the treated cells were pelleted and resuspended in lysis buffer. After measurement of protein concentrations, equal amounts of whole cellular extracts were analyzed on 10% SDS–PAGE and transferred to the nitrocellulose filter. For immunoblotting, the nitrocellulose filter was incubated with TBS-T containing 5% nonfat milk (milk buffer) for 2 hr, and then blotted with antisera against c-*jun* N-terminal kinase (JNK, Santa Cruz Biotechnology) for overnight at 4°. After washing with milk buffer twice, the filter was incubated with donkey antimouse IgG conjugated to horseradish peroxidase at a concentration of 1:5000 for 30 min. The filter was then incubated with the substrate and exposed to X-ray film.

2.8. Immunoprecipitation kinase assay

The immunoprecipitation kinase assay has been detailed somewhere [37]. The JNK substrate, GST-c-*jun* fusion protein, was a kind gift from Dr. S.-F. Yang (Academia Sinica). Myelin basic protein used as a substrate for extracellular signal regulated protein kinase (ERK) was purchased from Sigma. The antibodies for kinase assays were either kindly provided by Dr. Tse-Hua Tan (Baylor College of Medicine for JNK) or purchased from Santa Cruz Biotechnology (for ERK). To perform the assay, the whole cellular extract 30 μg was incubated with 3 μL of specific antibody in incubation buffer containing 25 mM HEPES (pH 7.7), 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 μM leupeptin and 400 μM PMSF for >2 hr. The mixture was then immunoprecipitated by addition of protein A beads and rotated at 4° overnight. After extensive wash, twice with HEPES washing buffer containing 20 mM HEPES (pH 7.7), 50 mM $MgCl_2$, 0.1 mM EDTA and 0.05% Triton X-100, twice with LiCl washing buffer containing 500 mM LiCl, 100 mM Tris (pH 7.6), 0.1% Triton X-100 and 1 mM DTT and twice with kinase buffer containing 20 mM MOPS (pH 7.2), 2 mM EDTA, 10 mM $MgCl_2$, 0.1% Triton X-100 and 1 mM DTT, the beads were resuspended in 40 μL kinase buffer with addition of cold ATP (250 μM), substrates and 10 μCi of [γ-^{32}P]ATP. The mixture was incubated at 30° with occasional gentle mixing for 30 min. The reaction was then terminated by resuspending in 1% SDS solubilizing buffer and boiled for 5 min and analyzed in SDS–PAGE.

2.9. Transfection assays

The transfection assays were performed according to our previous work [36] with some modifications. In order to reduce cell damage, we used the transfection reagent TransFast™ (Promega) to transfect plasmids into cells instead of using electroporation. In brief, 1×10^6 human leukemic T cell line Jurkat were evenly mixed with 2 μg of reporter plasmid pNF-κB-Luc or pAP-1-Luc (Stratagene) and TransFast™ transfectant (6 μL) in triplicate. Forty-eight hours after transfection, the cells were equally distributed and pretreated or not with various dosages of EGb and then stimulated with or without PMA (5 ng/mL) and ionomycin (1 μM) for 24 hr. Subsequently, the cell pellets were collected, the total cell lysates prepared and the luciferase activities were determined according to manufacturer's instructions (Promega).

2.10. Statistics

The results are expressed as means ± SD. A paired or unpaired Student's *t*-test was used to determine the significance of differences; a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. EGb effects on cytokine production

Because cytokines play important roles in atherosclerosis and ischmemia/reperfusion myocardial and cerebral injuries, within noncytotoxic concentrations, we examined the effects of EGb on the cytokine production from activated T cells. As suggested, we used PMA + ionomycin stimulation to mimic the situation of T cell activation [38]. In the presence of EGb, the production of several cytokines, including IL-2, IL-4, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) induced by PMA + ionomycin stimulation, was greatly inhibited in a concentration-dependent manner (Fig. 1).

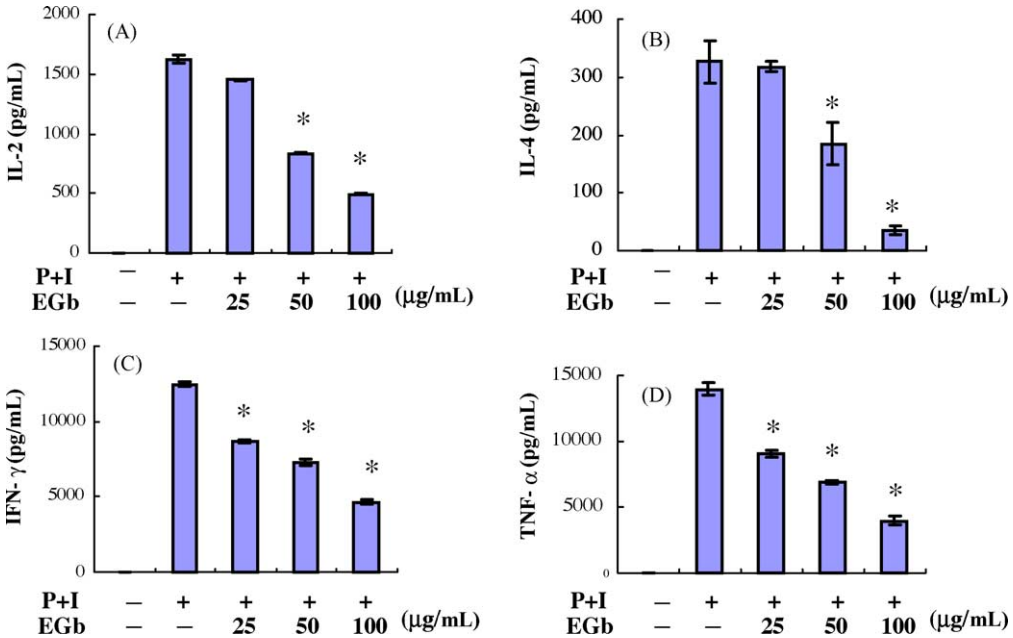

Fig. 1. Effects of EGb on PMA + ionomycin-induced cytokine production from T cells. Human peripheral blood T cells at 1×10^6/mL were pretreated with various concentrations of EGb for 2 hr and then stimulated with PMA + ionomycin for another 24 hr. The supernatants were collected for IL-2 (A), IL-4 (B), IFN-γ (C) and TNF-α (D) measurements. The representative data out of at least six different donor cells with similar results are shown as means ± SD. Asterisk (∗) denotes statistical significance ($P < 0.05$) as compared to the stimulated one in the absence of EGb treatment.

When anti-CD3 + anti-CD28 mAbs were used as a more physiological stimuli to treat T cells, we also observed the inhibition of cytokine production by EGb (Fig. 2). Although these cytokines were susceptible to the inhibition by EGb, some difference existed; for example, EGb at 25 μg/mL significantly inhibited the PMA + ionomycin-induced production of IFN-γ and TNF-α, however, this concentration only insignificantly reduced the production

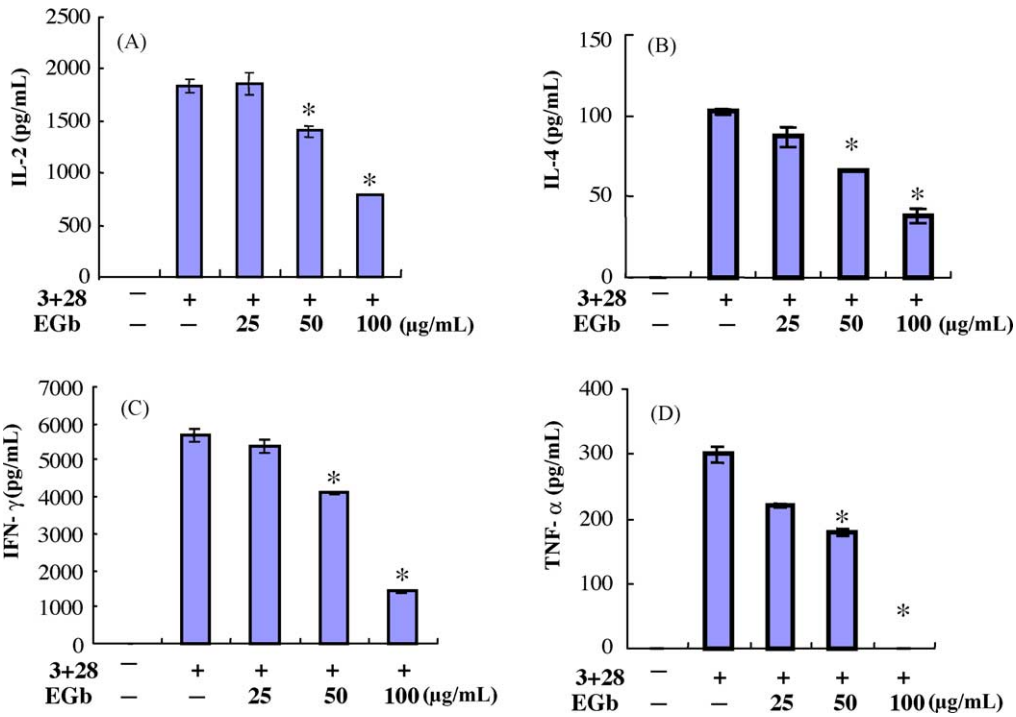

Fig. 2. Effects of EGb on anti-CD3 + anti-CD28 mAbs-induced cytokine production from T cells. Human peripheral blood T cells at 1×10^6/mL were pretreated with various concentrations of EGb for 2 hr and then stimulated with anti-CD3 + anti-CD28 mAbs for another 24 hr. The supernatants were collected for IL-2 (A), IL-4 (B), IFN-γ (C) and TNF-α (D) measurements. The representative data out of three different donor cells with similar results are shown as means ± SD. Asterisk (∗) denotes statistical significance ($P < 0.05$) as compared to the stimulated one in the absence of EGb treatment. 3 + 28 stands for anti-CD3 + anti-CD28 mAbs stimulation.

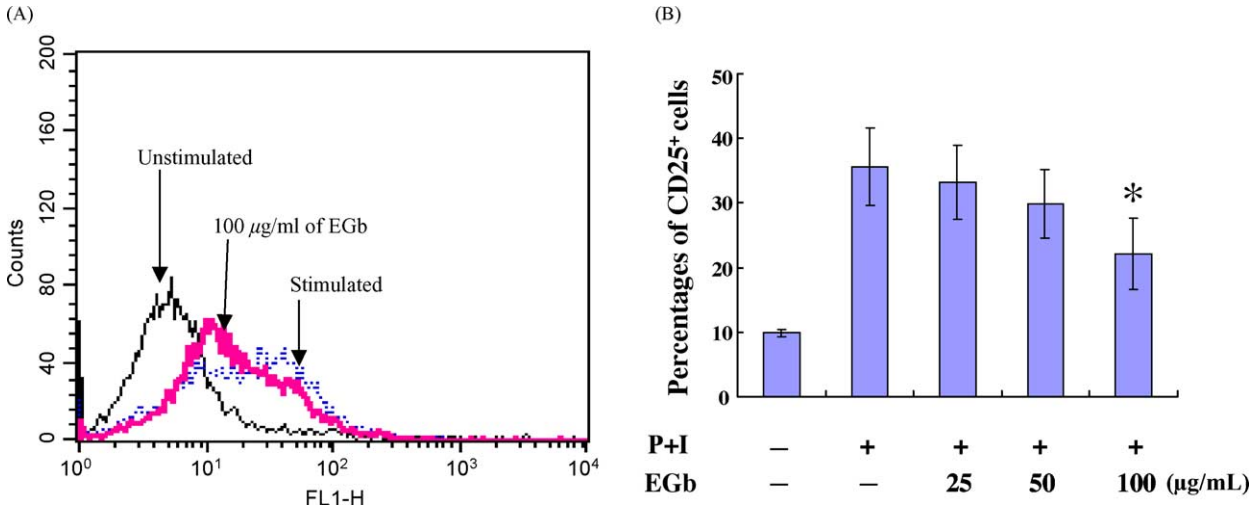

Fig. 3. EGb inhibits the expression of CD25 (IL-2Rα) on activated T cells. Human peripheral blood T cells at 1×10^6/mL were treated with 100 μg/mL (A) or various concentrations (B) of EGb for 2 hr and then stimulated or not with PMA + ionomycin for another 24 hr. The expression of CD25 was measured by a flow cytometer. The representative data (A) and means ± SD (B) out of at least six different donor cells are shown. Asterisk (∗) denotes statistical significance ($P < 0.05$) as compared to the stimulated one in the absence of drug treatment.

of IL-2 and IL-4 (Fig. 1). In contrast, EGb at 25 μg/mL did not significantly inhibited anti-CD3 + anti-CD28 mAbs-induced IL-2, IL-4, TNF-α and IFN-γ production (Fig. 2). This indicates that the molecules involved in individual stimuli-triggered signal transduction pathways leading to the production of IL-2, IL-4, TNF-α and IFN-γ may show differential susceptibility to EGb inhibition.

3.2. EGb effects on activation marker expression

Since substantial amount of T cells in cardiovascular and cerebrovascular diseases has been shown to be in activated status with the expression of activation markers, we then determined if EGb could also affect the expression of T cell activation markers. As shown in Fig. 3, EGb, at the highest concentration used, effectively reduced the expression of T cell activation marker CD25.

3.3. EGb down-regulated AP-1 but not NF-κB DNA-binding activity

We next examined the effects of EGb on the activation of transcription factors NF-κB, a family of proteins extensively involved in regulation of a variety of cytokine genes. After stimulation with PMA + ionomycin, peripheral blood T cell nuclear extracts contained strong NF-κB DNA-binding activity (Fig. 4A). Unexpectedly, EGb had limited or no inhibitory effects on NF-κB DNA-binding activity (Fig. 4A). We then examined another potential EGb targets, the AP-1 transcription factors that also bind the enhancer/promoter regions of many cytokine genes in activated T cells [39]. As shown in Fig. 4B, in the presence of EGb, the PMA + ionomycin-induced AP-1 DNA-binding activity was greatly suppressed. To be more physiological, we chose anti-CD3 + anti-CD28 mAbs to stimulate T cells. By EMSA analysis, we showed that EGb effectively down-regulated CD28-costimulated AP-1 DNA-binding activity (Fig. 4C). By competition assays, we demonstrated that the induced nuclear protein complex bound to radiolabeled AP-1 oligonucleotides could be blocked by wild-type but not mutant AP-1 oligonucleotides (Fig. 4D). This confirmed the nature of the shifted band in EMSA to be AP-1 complex. Furthermore, when EGb was present 60 min before the addition of stimuli, it effectively inhibited AP-1 DNA-binding activity. However, the inhibitory effect was totally abolished when EGb was added 30 min after the addition of stimuli (Fig. 4E). These results suggest that EGb may target certain molecules along the AP-1 activation pathway and the preinactivation of these molecules before the initiation of stimulation signal was necessary for EGb to block PMA + ionomycin-induced AP-1 activation.

3.4. EGb blocked JNK but not ERK activity

The inhibition of AP-1 DNA-binding activity by EGb suggests that this drug may have some effects on the AP-1 upstream protein kinase, JNK that phosphorylates c-*jun* and activates its transcriptional activity. To answer this question, the immunoprecipitation kinase asssys were performed. As shown in Fig. 5A and B, PMA + ionomycin treatment induced a transient activation of JNK activity. The peak effect was found at 15 min after stimulation. In the presence of EGb pretreatment, the stimuli-induced JNK activity was greatly reduced. By Western blotting, we showed that the inhibition was not due to its effects on JNK protein levels (Fig. 5C). Concurrently, the examination of another mitogen-activated protein kinase, ERK, activity revealed that EGb had very limited or no inhibitory effects on this kinase (Fig. 5D).

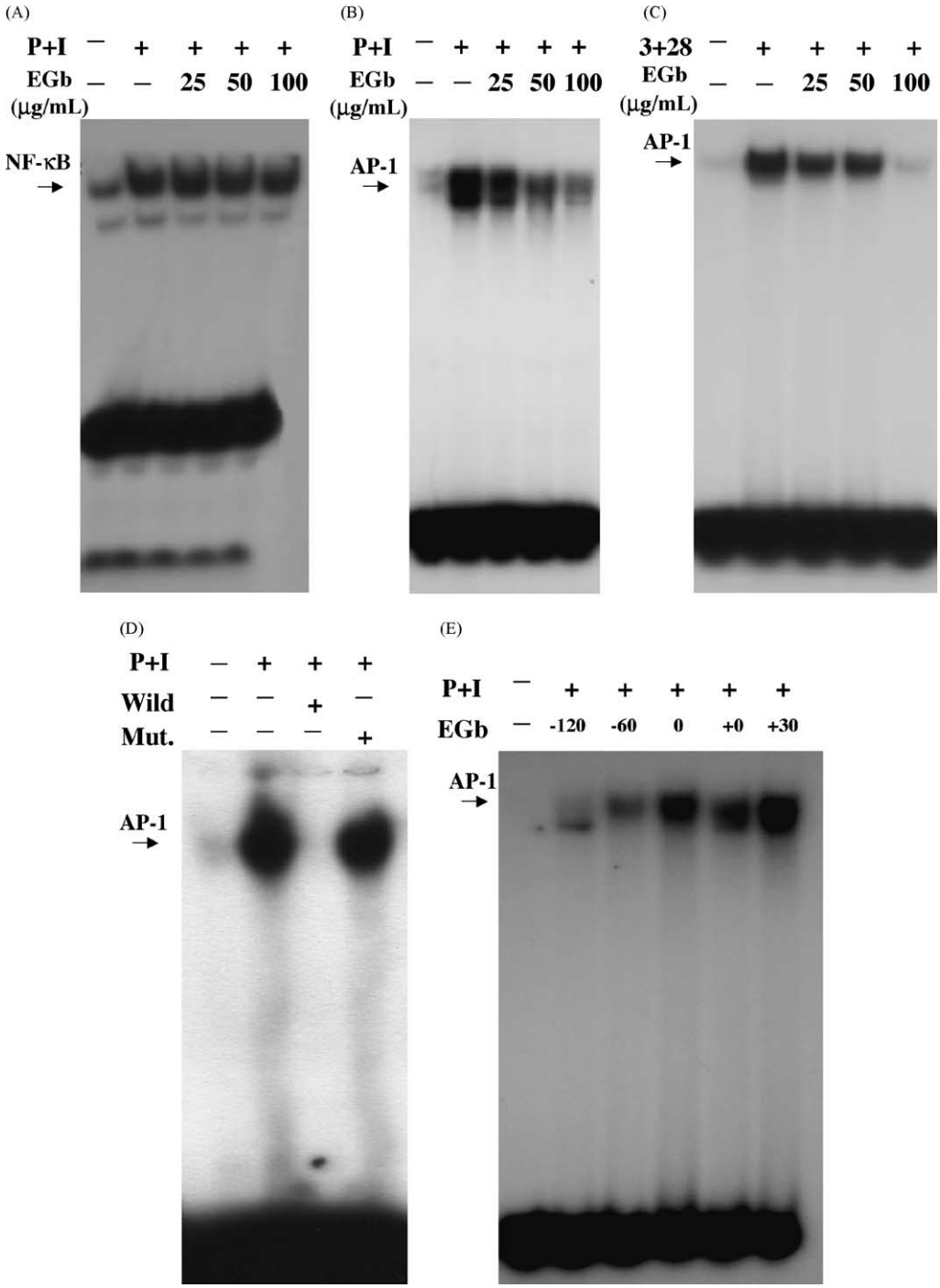

Fig. 4. EGb blocks AP-1, but not NF-κB, DNA-binding activity. Human peripheral blood T cells at 2×10^6/mL were pretreated with various concentrations of EGb for 2 hr and then stimulated or not with PMA + ionomycin (A, B, D and E) for 60 min or anti-CD3 + anti-CD28 (C) for 14 hr. The nuclear extracts containing 5 μg of total protein from treated cells were analyzed by EMSA. The ^{32}P-labeled oligonucleotides containing the κB (A) or AP-1 (B–E) site were used as probes. The whole reaction mixtures were incubated for 20 min and then analyzed on a 6.6% native polyacrylamide gel. A competition study was done with 100-fold molar excess of unradiolabeled wild-type (Wild) or mutant (Mut.) AP-1 oligonucleotides (D). The competitors were preincubated with nuclear extracts for 10 min before the addition of the radiolabeled AP-1 probe. In (E), the cells were pretreated 120 (−120) or 60 (−60) min with EGb before the addition of the stimuli or treated 0 (+0, both drug and stimulus were added at the same time) or 30 (+30) min after the addition of the stimuli. The representative data are shown. 3 + 28 stands for anti-CD3 + anti-CD28 mAbs stimulation.

3.5. EGb inhibited AP-1 but not NF-κB transcriptional activity in vivo

To further investigate whether the inhibition of AP-1 activity by EGb *in vitro* could also be seen *in vivo*, we transiently transfected AP-1-luciferase or NF-κB-luciferase reporter plasmids into human leukemic T cell line Jurkat. Forty-eight hours after transfection, the cells were treated with EGb at various dosages and then were stimulated with PMA + ionomycin to induce NF-κB or AP-1 transcriptional

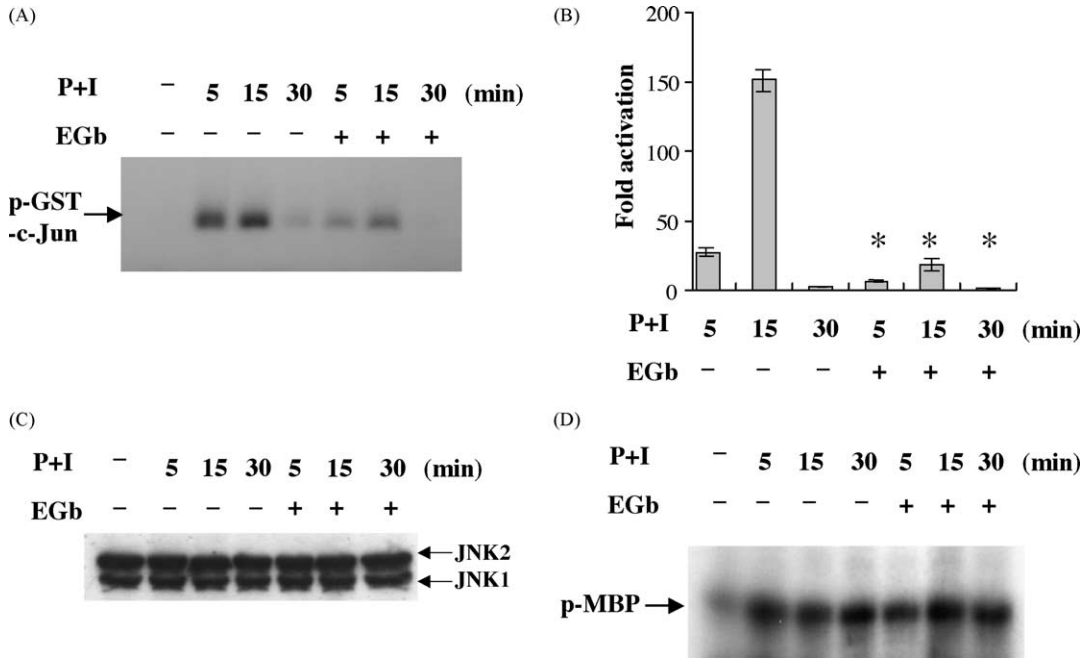

Fig. 5. EGb inhibits JNK but not ERK activity. Human peripheral blood T cells at 2×10^6/mL were pretreated or not with 100 μg/mL of EGb for 2 hr and then stimulated with PMA + ionomycin for various time points. After wash, cell pellets were collected and equal amounts of whole cell lysates were immunoprecipitated with anti-JNK (A) or anti-ERK antibody (D). After sequential wash, the substrates (GST-c-*jun* for JNK and myelin basic protein for ERK) and 10 μCi of [γ-^{32}P]ATP were added. After kinase reaction, the reaction mixture were analyzed. For determination of the JNK protein level, the Western blotting assays were performed as described in Section 2 (C). The representative data (A, C and D) and the statistical analysis of the results for JNK from three different donors (B) are shown. Asterisk (*) denotes statistical significance ($P < 0.05$) as compared with cells stimulated in the absence of EGb in indicated parallel time points. p-MBP stands for phosphorylated myelin basic protein.

activity. In consistent with *in vitro* observations, EGb significantly inhibited the transcriptional activity of AP-1 (Fig. 6A). In contrast, EGb did not affect or mildly-induced NF-κB transcriptional activity *in vivo* (Fig. 6B). The results of EMSA analysis in Jurkat T cells were also consistent with those performed in human peripheral blood T cells (Fig. 6C and D).

4. Discussion

In the present study, we explored the possible effects and mechanisms of EGb in activation of human peripheral blood T cells, an important population among immune effector cells in inflammatory responses of cardiovascular and cerebrovascular diseases. We concluded that EGb efficiently blocked several cytokines, including IL-2, IL-4, IFN-γ and TNF-α production from activated human peripheral blood T cells (Figs. 1 and 2). The inhibition is likely to be mediated through the down-regulation of JNK-AP-1 signaling pathway (Figs. 4 and 5). The observations shown in *in vitro* studies were also demonstrated in *in vivo* studies as EGb potently inhibited the transcriptional activation of the AP-1-dependent reporter gene (Fig. 6). Interestingly, EGb had no or very limited effects on ERK and NF-κB activities (Figs. 4–6). This may suggest a relative specificity of EGb in blocking T cell activation.

Given the importance of cytokines in causing cardiovascular and cerebrovascular damages, the inhibition of cytokine production by EGb is likely to be clinically relevant. Serum cytokines like IL-2 and TNF-α have been demonstrated to deliver negative inotropic effects on cardiac papillary muscles through nitric oxide-dependent mechanisms [40]. In association with the cardiovascular and cerebrovascular pathophysiology, TNF-α that shares several activities with IFN-γ, has been shown to induce the expression of adhesion molecules on endothelial cells, to increase the synthesis of matrix metalloproteinases that favor vascular matrix degradation as well as to up-regulate the expression of the scavenger receptor in smooth muscle cells [41,42]. In addition, TNF-α can induce the production of reactive oxygen species in tissue cells like endothelial cells and activate oxidative stress-responsive genes [43]. The TNF-α-mediated endothelial damage may finally result in vascular calcification [44]. Convincingly, anti-TNF-α therapy has been shown to improve myocardial recovery in cardioplegia-induced ischemia in rats [45]. Whereas the combination of the effects of several cytokines may give further detrimental consequences through synergism, the broad-spectrum inhibition of cytokine production from T cells by EGb provides additional therapeutic benefits. This suggestion is also supported by the evidence that AP-1 regulates the expression of several inflammatory cytokine genes [46]. Interestingly, *via* blocking AP-1 activity, transforming growth factor-beta 1 effectively inhibited the

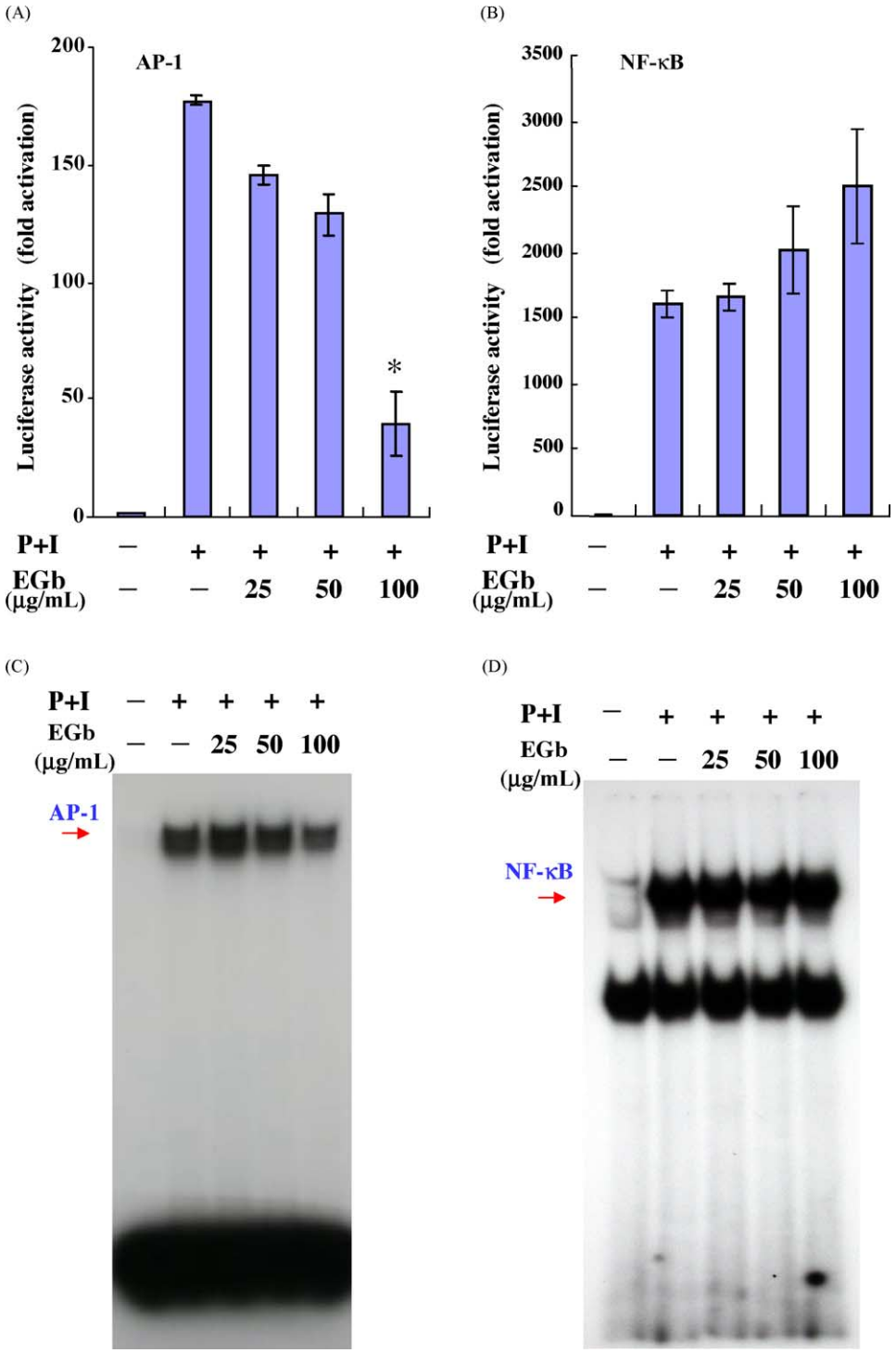

Fig. 6. EGb suppressed transcriptional activity of AP-1 but not NF-κB *in vivo*. Human leukemic T cell line Jurkat at 1×10^6/mL were mixed together with pAP-1-Luc (A, labeled as AP-1) or pNF-κB-Luc (B, labeled as NF-κB) reporter plasmids and the transfection reagent TransFast™. Forty-eight hours after transfection, the cells were pretreated with EGb at various dosages for 2 hr. After stimulation with PMA (5 ng/mL) + ionomycin (1 μM) for another 24 hr, cells were collected and the total cell lysates were analyzed for luciferase activities. Values are expressed as fold induction of luciferase activity when compared with unstimulated cells. The data shown (A and B) are from at least three independent experiments performed. Asterisk (*) denotes statistical significance ($P < 0.05$) as compared with cells stimulated in the absence of EGb treatment. The EMSA analysis in Jurkat T cells were performed exactly as described in Fig. 4. The representative data (C and D) are shown.

cytokine-mediated induction of metalloelastase in macrophages [47]. Because AP-1 can also be induced by a variety of mitogens, cytokines and pathogens, EGb may have therapeutic potential in diseases driven by activated T cells.

The AP-1 is a series of related dimeric protein complexes that compose of members of Jun and Fos that mediate diverse signaling events [48,49]. While the regulation of AP-1 activity is complex, there are several

kinases and upstream kinase kinases involved in AP-1-mediated signaling pathways. In the present study, we demonstrated that EGb down-regulated JNK but had limited effects on ERK activities (Fig. 5). This observation is consistent with the notion that in living cells, the phosphorylation of the N-terminal sites of c-*jun* parallels the activation of JNK and does not correlate with ERK activation [50]. Both JNK and ERK belonging to a family of mitogen-activated protein kinases (MAP kinases) are activated by dual phosphorylation at the tripeptide motif Thr-Xaa-Tyr. Although the phosphorylation motif only shows subtle difference, the ERK is activated by the MAP kinase kinases (MKKs) MKK1 and MKK2 and the JNK is activated by MKK4 and MKK7 [51,52]. The MKKs are activated by MKK kinases and whose activation are dependent on the more upstream singnaling pathways. In contrast to the activation of ERK by Ras, JNK is activated by Rho family GTPases, including Rac and Cdc42. Because only MAP kinase level was examined in this study, it is possible that the target for EGb may be more upstream or close to the cell membrane. Interestingly, the inhibition of JNK-AP-1 activity as well as TNF-α production by EGb was also observed by other researchers using laboratory mice and murine macrophage cell line as the study models [53]. However, probably because of the different stimuli and different tissue cell systems examined, ERK appeared to be susceptible in macrophages but not in T cells to the inhibition by EGb ([53] and this study). Given the complexity of the molecules involved in JNK and ERK signaling pathways, at this stage, the present study can only speculate and does not conclude the effect of EGb on these two signaling pathways.

According to the published literatures, although it has rarely or never been investigated in T cells before this study, both antioxidative and antiapoptotic effects of EGb shown in the examination of other tissue cells and in animals may be related to the observed mechanisms demonstrated in this study [24,31]. Oxidative stress has been well recognized as an activator of JNK-AP-1 signaling pathway (reviewed in [54]). Thus, the blockage of JNK-AP-1 signaling pathway by EGb may explain in part its antioxidative effects. The induction of cellular apoptosis by various agents also requires the activation of JNK and the blockage of JNK activation suppresses apoptotic effects [55,56]. Therefore, the inhibition of JNK activation by EGb may be correlated with its potential antiapoptotic effects. Nevertheless, further work is required to delineate these interesting relationships.

One of the major concerns about this study is the physiological relevance of the concentrations of EGb examined. Through liquid chromatography/atmospheric pressure chemical ionization mass spectrometry analysis, the peak concentrations of ginkgolides and bilobalide, two of the major constituents of EGb, were examined in volunteers taking EGb [57]. It reveals that, dependent on the forms of EGb administered, maximum plasma concentrations of total ginkgolides and bilobalide are 85.0 and 181.8 μg/mL for free and phospholipid complex forms, respectively. Accordingly, the effects of EGb with concentrations ranging from 25 to 100 μg/mL examined in this study should have their clinical relevance.

The limitation of this study is that we only showed *in vitro* EGb effects on human peripheral blood T cells isolated from healthy donors although we found EGb could also down-regulate AP-1 transcriptional activity in Jurkat T cells. The analysis of T cells in EGb-treated disease models should provide a much stronger support for our conclusions. Nevertheless, based upon our observations in peripheral blood T cells, more basic and clinical trials on EGb for cardiovascular and cerebrovascular diseases are expected to be coming out in the near future.

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