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Pseudolaric Acid B Suppresses T Lymphocyte Activation Through Inhibition of NF-κB Signaling Pathway and p38 Phosphorylation

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ABSTRACT

Pseudolaric acid B (PAB) is a major bioactive component of the medicinal plant *Pseudolarix kaempferi*. Traditional medicine practitioners in Asia have been using the roots of this plant to treat inflammatory and microbial skin diseases for centuries. In the current study, in vitro immunosuppressive effect of PAB and the underlying mechanisms have been investigated. The results showed that PAB dose-dependently suppressed human T lymphocyte proliferation, IL-2 production and CD25 expression induced by co-stimulation of PMA plus ionomycin or of anti-OKT-3 plus anti-CD28. Mechanistic studies showed that PAB significantly inhibited nuclear translocation of NF-κB p65 and phosphorylation and degradation of IκB-α evoked by co-stimulation of PMA plus ionomycin. PAB could also suppress the phosphorylation of p38 in the MAPKs pathway. Based on these evidences, we conclude that PAB suppressed T lymphocyte activation through inhibition of NF-κB and p38 signaling pathways; this would make PAB a strong candidate for further study as an anti-inflammatory agent. J. Cell. Biochem. 108: 87–95, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: PSEUDOLARIC ACID B; T LYMPHOCYTE ACTIVATION; CD25; NF-κB; p38

Pseudolaric acid B (PAB) is one of the major chemical components of the root bark of the medicinal plant *Pseudolarix kaempferi* (Fig. 1A). This plant has long been used as an anti-inflammatory and anti-fungal remedy in traditional Chinese medicine for treating skin inflammation [Li et al., 1995]. Previous pharmacological studies have shown that PAB inhibits the growth of a number of human cancer cell lines, such as KB, A-549, HCT-8, P-388, and L-1210 in vitro [Pan et al., 1990]. PAB also induces cell cycle arrest at G2-M transition, leading to apoptosis and disruption of the cellular microtubule networks, in turn inhibiting the formation of mitotic spindles [Wong et al., 2005]. In addition, PAB demonstrates significant anti-angiogenic effect on human microvessel endothelial cells through inhibition of cell proliferation, migration, and tube formation [Tong et al., 2006]. However, so far, the effect of PAB on human T lymphocyte activation has not been reported.

NF-κB, a transcriptional factor composed of mainly p105/p50 (NF-κB1), p100/p52 (NF-κB2), p65(Rel A), Rel B, and c-Rel, is involved in the expression of numerous components of the human immune system including production of cytokines, chemokines, and adhesion molecules [Li and Verma, 2002]. NF-κB also contributes to the development and survival of cells and tissues that regulate the normal immune responses. Meanwhile, T lymphocytes dominate the autoimmune responses in the human body, while their proliferation relies on NF-κB activation. NF-κB activation can also facilitate production of a series of pro-inflammatory cytokines and then potentiate the upset and progression of inflammatory and autoimmune conditions [Hayden et al., 2006]. Thus, NF-κB signaling pathway in T lymphocytes has become a central focus of clinical interventions and the most attractive therapeutic target for developing new drugs to treat inflammatory and autoimmune diseases [Calixto et al., 2003; Gilmore, 2006].

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine protein kinases that include the extracellular-signal-regulated kinase (ERK), the Jun N-terminal kinase (JNK) and p38 kinase, all of which are highly conserved across eukaryotic species. MAPKs play important roles in cellular processes, such as proliferation, stress responses, and apoptosis [Liu et al., 2007], and

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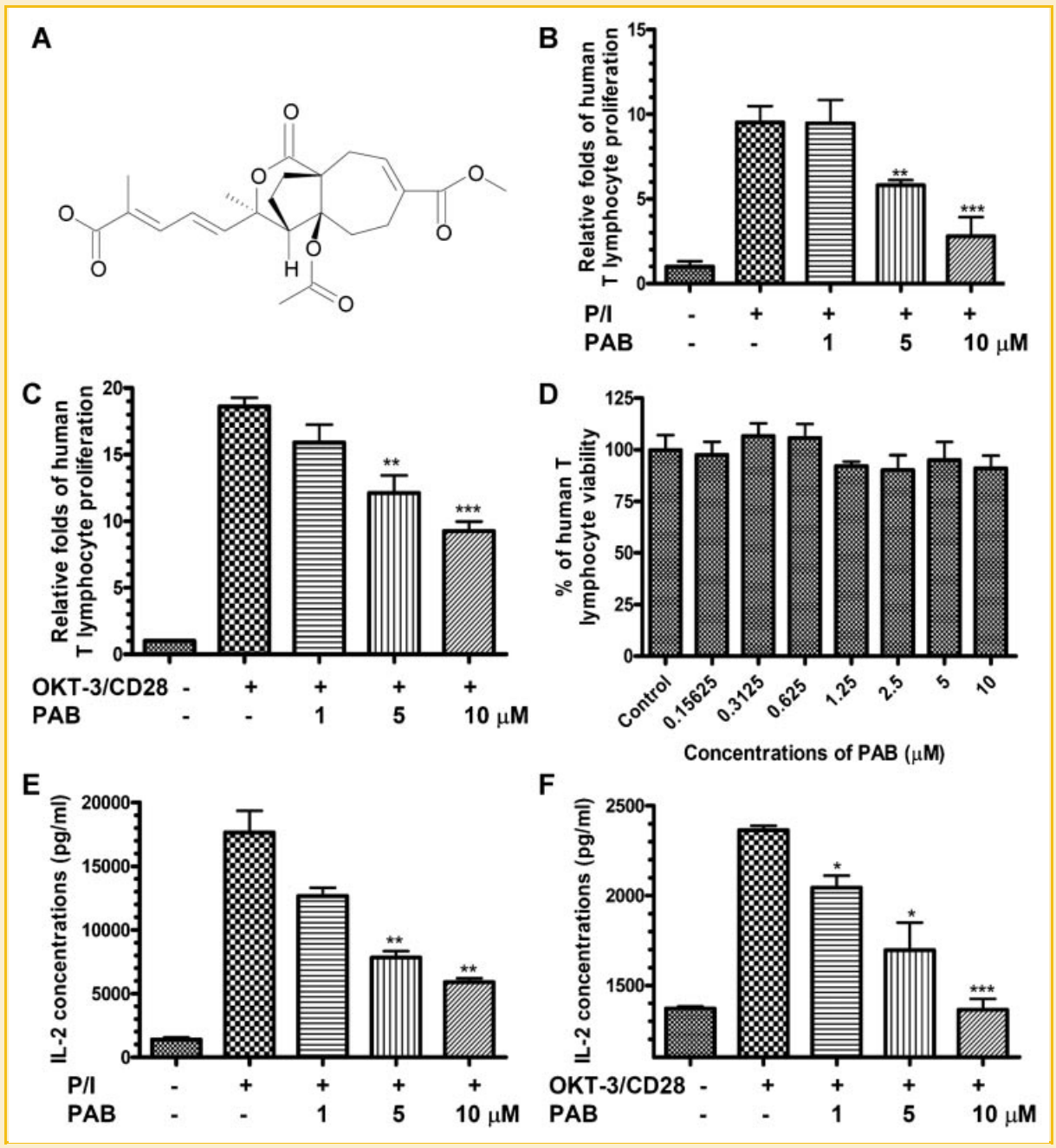

Fig. 1. Effects of pseudolaric acid B on human T lymphocyte proliferation and IL-2 production. A: The chemical structure of pseudolaric acid B ($C_{23}H_{28}O_8$, PAB). B,C: Effect of PAB on human T lymphocyte proliferation co-stimulated with PMA plus ionomycin or anti-OKT-3 plus anti-CD28. The cells (10^5/well) were pre-treated with indicated concentrations of PAB for 2 h and then activated with 20 ng/ml PMA plus 1 μM ionomycin (P/I) for 72 h in the cell culture (B). The cells (10^5/well) were stimulated with the immobilized anti-OKT-3 (5 μg/ml) plus anti-CD28 (1 μg/ml) (OKT-3/CD28) in presence or absence of PAB for 72 h in the cell culture (C). BrdU was added to the cells for 14 h incubation before the end of cell culture, and then the amount of BrdU incorporation was measured by using plate reader at 450 nm. Data are expressed as the relative fold of BrdU incorporation of the control cells and represents the mean ± SEM of three independent experiments. D: Cytotoxicity examination of PAB. The cells (10^5/well) isolated from the human buffy-coat blood as mentioned method were treated with PAB at indicated concentrations for 3 days, and then MTT reagent was added to the cells for 4 h of incubation followed by addition of the solubilization buffer. The absorbance was then read at 570 nm. Data are expressed as the percentage of absorbance of control cells and represents the mean ± SEM of three independent experiments. E,F: Effect of PAB on IL-2 secretion of the cells stimulated by PMA plus ionomycin or anti-OKT-3 plus anti-CD28. The cells (10^5/well) were firstly treated with PAB (1, 5, or 10 μM) for 120 min and then stimulated with 20 ng/ml PMA plus 1 μM ionomycin for 48 h (E). The cells (10^5/well) were stimulated with immobilized anti-OKT-3 (5 μg/ml) plus anti-CD28 (1 μg/ml) in absence or presence PAB for 48 h (F). IL-2 concentrations in cell culture supernatants were determined by ELISA method. Data reported represent the mean ± SEM of three independent experiments. Significance of differences shows as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

they are involved in all aspects of immune responses from the innate to the adaptive, from the initiation of immune responses to the activation-induced cell death [Dong et al., 2002].

In the current study, we aimed to examine the effects of PAB on human T lymphocyte activation through NF-κB and MAPKs signaling pathways. The results showed that PAB could significantly suppress human T lymphocyte activation induced by PMA plus ionomycin or by anti-OKT-3 plus anti-CD28 co-stimulation. This suppression was evidenced in the inhibition of cell proliferation, decreased IL-2 production and suppressed expression of T cell activation markers. Further mechanistic study demonstrated that the inhibitory effect of PAB is closely associated with down-regulation of NF-κB and p38 MAPKs signaling pathways.

MATERIALS AND METHODS

DRUGS AND REAGENTS

Pseudolaric acid B of >98% purity verified by HPLC was obtained from Merck & Co (Rahway, NJ). The anti-OKT-3 and anti-CD28 used for stimulation of human T lymphocytes were purchased from eBioscience and BD Pharmingen (San Diego), respectively. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were obtained from Sigma Chemical Co. (St. Louis, MO) and Calbiochem (San Diego), respectively.

CELL CULTURE AND STIMULATION

Human peripheral blood T lymphocytes were isolated from buffy coat blood, based on the method described in a previous report [Ghittoni et al., 2005]. In brief, the buffy coat blood obtained from the Red Cross Association of Hong Kong was mixed with normal saline, and then transferred to Ficoll-Pague (Amersham Biosciences) in 50 ml tubes. The mixture was centrifuged at 400*g* for 35 min to separate the blood into layers. The layer of the non-adherent mononuclear cells was collected, from which the macrophages were removed by adherence to obtain T lymphocytes. And then, the non-adherent cells containing dominantly the T lymphocytes, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The purity of CD3+ T cells in non-adherent cells has been determined by flow cytometry and the purity is around 70% in the non-adherent cells. To stimulate T lymphocyte activation, two sets of co-stimulators, that is, 20 ng/ml PMA plus 1 μM ionomycin or immobilized anti-OKT-3 mAb (5 μg/ml) plus anti-CD28 mAb (1 μg/ml), were used. According to different purposes of the experiments, one set of co-stimulators from the above two was employed in each experiment.

T CELL PROLIFERATION ASSAY

The isolated cells (1×10^6/ml) were cultured in 96-well plates in triplicate in 100 μl of RPMI 1640 medium plus 10% FBS. The cells were then stimulated with 20 ng/ml PMA plus 1 μM ionomycin or immobilized anti-OKT-3 (5 μg/ml) plus anti-CD28 (1 μg/ml) in the presence or absence of PAB for 72 h. BrdU (Roche) at final concentration of 10 μM was added to the cells, and they were incubated for another 14 h. Active dividing cells incorporate more BrdU in their DNA; thus, the degree of cell proliferation can be detected by quantification of BrdU incorporation. In the current experiments, BrdU was determined by ELISA method according to the manufacturer's instruction. Data were obtained from three independent experiments.

CYTOTOXICITY ASSAY

Cytotoxicity of PAB was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described previously [Gerlier and Thomasset, 1986]. Briefly, the isolated cells (1×10^5/well) were cultured in triplicate in a 96-well plate in 100 μl of RPMI 1640 medium plus 10% FBS for 72 h. MTT (5 mg/ml) was added for 4 h incubation, and then a solvent (10% sodium dodecyl sulfate (SDS), 50% N,N-dimethy formamide, pH 7.2) was added to dissolve the purple precipitate. A_{570nm} was determined from each well on the next day. The percentage of cell viability was calculated using the following formula: Cell viability (%) $= A_{treated}/A_{control} \times 100$. Data reported represent three independent experiments.

ENZYME-LINKED IMMUNOSORBENT ASSAY

The level of IL-2 secreted by the activated human T lymphocytes in the designed cell cultures was evaluated by using IL-2 human enzyme-linked immunosorbent assay (ELISA) method (BD Pharmingen). In brief, the cells (1×10^5/well) were incubated in presence or absence of PAB for 2 h at different concentrations, and then the cells were stimulated with 20 ng/ml PMA plus 1 μM ionomycin for 48 h. Using co-stimulator of 5 μg/ml immobilized anti-OKT-3 plus 1 μg/ml anti-CD28, the cells were cultured in presence or absence of PAB for 48 h on the plate that was pre-coated with these two antibodies. The cell-free culture supernatants were collected, and then the concentrations of IL-2 in the supernatants were determined by ELISA method according to the manufacturer's instruction. All samples were determined in triplicate. Data were obtained from three independent experiments.

T CELL SURFACE MARKER ANALYSIS

Expression of T lymphocyte surface markers, CD25 and CD69, was evaluated by flow cytometry based on the previously described method [Leung et al., 2005]. The cells (1×10^6/well) were pre-treated with PAB for 2 h and then stimulated with PMA (20 ng/ml) or PMA (20 ng/ml) plus ionomycin (1 μM) [Leung et al., 2005]. The expressions of CD69 and CD25 on the cell surfaces were determined after 24 and 48 h of cell culture, respectively. At the end of cell culture, the cells were harvested and washed with PBS. Cells were then stained with specific antibodies in the combination of either anti-CD69-FITC and anti-CD3-PE or anti-CD25-FITC and anti-CD3-PE (BD Pharmingen) for 30 min at room temperature in dark, and then fixed with 4% paraformaldehyde (PFA). On the following day, samples were analyzed on FACS Calibur Flow Cytometer using CellQuest software (BD Biosciences, San Diego). The compensation standards were composed of the separate tubes of cells stained with positive single-color antibodies for each of the flourochromes.

ANALYSIS OF NUCLEAR AND WHOLE CELLULAR PROTEINS BY USING WESTERN BLOTTING

For NF-κB nuclear translocation assay. The cells (6×10^6/well) were pretreated with PAB in different concentrations for 60 min and then

the cells were stimulated with 20 ng/ml PMA plus 1 μM ionomycin for 120 min. After treatment, the cells were harvested and washed with PBS twice. Nuclear proteins of the cells were then prepared using NucBuster™ Reagents (Novagen) according to manufacturer's instruction. In brief, the washed cells were resuspended using 60 μl NucBuster™ Reagent 1 per 30 μl of the packed cells and processed twice by vortexing for 15 s, incubated on ice for 5 min, vortexed again for 15 s, and finally centrifuged at 16,000*g* for 5 min. The supernatants, which contained cytoplasmic proteins, were discarded; the cell pellets were resuspended in 30 μl of NucBuster Extraction Reagent 2. The same vortexing, icing, and repeated vortexing procedures were repeated once to prepare nuclear proteins of the cells. Equal amounts of nuclear proteins were analyzed by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and then the proteins were electro-transferred onto nitrocellulose membrane. The nitrocellulose membrane was blocked by 5% dried milk for 60 min and then was incubated with NF-κB p65 antibody overnight at 4°C. On the next day, the membrane was washed with TBS-T again and further incubated with HRP-conjugated secondary antibodies for 60 min. The blots were developed using ECL Western Blotting Detection Reagents (Amersham Biosciences).

For detection of IκBα degradation and the phosphorylation forms of JNK (P-JNK), total JNK; phosphorylation forms of ERK1/2 (P-ERK1/2), total ERK1/2, phosphorylation forms of p38 (P-p38) and total p38 kinase from whole cellular proteins, the cells (4×10^6/well) were pre-treated with different concentrations of PAB (1–10 μM) for 60 min. And then, they were incubated with 20 ng/ml PMA plus 1 μM ionomycin for another 10 min (for detection of MAPKs) or 60 min (for detection of IκBα degradation), and finally harvested. In examination of the phosphorylation forms of IκBα, the cells (4×10^6/well) were pre-incubated with different concentrations of PAB (1–10 μM) together with 100 μM N-acetyl-leucyl-leucyl-norleucinal (ALLN) (Calbiochem) for 60 min, and then the cells were incubated with 20 ng/ml PMA plus 1 μM ionomycin for another 60 min and finally harvested. The harvested T cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% Triton, 50 mM NaF, 1 mM sodium orthovanadate, 0.5 mM PMSF and 1 × protease inhibitor mix (Roche)) to produce whole cellular proteins. The whole cellular proteins (50 μg) were then subjected to electrophoresis in 10% SDS/PAGE and to immunoblotting as mentioned above. The primary antibodies used in this study were rabbit antibodies specific for IκBα, p65, and P-IκBα^{ser32} (Calbiochem) and mouse antibodies specific for actin and p38 (Santa Cruz), P-JNK (Thr183/Try185), JNK, P-ERK1/2 (Thr220/Try204), ERK, and P-p38 (Thr180/Try182) (Cell Signaling).

STATISTICAL ANALYSIS

Results are expressed as means ± SEM. One-way ANOVA or unpaired Student's *t*-test was used to determine the significance of difference; a value of $P < 0.05$ was considered statistically significant.

RESULTS

PAB INHIBITS CELL PROLIFERATION AND IL-2 PRODUCTION INDUCED BY EITHER PMA PLUS IONOMYCIN OR ANTI-OKT3 PLUS ANTI-CD28

Cell proliferation is one of the most outstanding hallmarks of T lymphocyte activation. Thus, we firstly examined the effect of PAB on cell proliferation evoked by co-stimulation of either PMA plus ionomycin or of anti-OKT3 plus anti-CD28 by using the BrdU incorporation method. The results showed that BrdU uptake in the PMA plus ionomycin-stimulated T lymphocytes was obviously and dose-dependently suppressed by pre-treating the cells with PAB (Fig. 1B). The results also showed that PAB significantly inhibited cell proliferation induced by the co-stimulation of anti-OKT3 plus anti-CD28 in a dose-dependent manner (Fig. 1C). To determine whether this anti-proliferative effect of PAB on human T lymphocytes results from the cytotoxic effect of the drug, the cytotoxicity experiments were performed using the MTT method. The results clearly showed no marked cytotoxicity of PAB at the indicated drug concentrations, which covers the range of effective concentrations of PAB used in the anti-proliferation experiments (Fig. 1D). These results indicate that PAB suppressed the proliferation of T lymphocytes by inhibiting cell reproduction but not by killing them. Moreover, mechanistic studies indicated that the anti-proliferative effect of PAB on human T lymphocytes involved suppression of IL-2 secretion of the cells. As shown in Figure 1E,F, co-stimulation of PMA plus ionomycin or of anti-OKT3 plus anti-CD28 in T lymphocytes markedly increases production of IL-2, while pre-treating the cells with PAB at concentrations of 1, 5, and 10 μM could dose-dependently reduce the increase of IL-2 production of the cells evoked by co-stimulation of either with PMA plus ionomycin (Fig. 1E). Similarly, PAB could significantly reduce the IL-2 production of the cells induced by co-stimulation with anti-OKT3 plus anti-CD28 (Fig. 1F).

PAB INFLUENCES THE EXPRESSIONS OF CD25 AND CD69 IN HUMAN T LYMPHOCYTES

To further understand the underlying mechanisms by which PAB suppresses the activation of human T lymphocytes, expression of CD69 and CD25 (IL-2 α receptor), which represents cell surface markers at the early and late stages of T lymphocyte activation respectively [Caruso et al., 1997; Lin and Leonard, 1997; Camargo et al., 2009], was examined by using flow cytometry analysis. The results demonstrated that both CD69 and CD25 antigens were expressed at very low levels (6.5% and 0.7%, respectively) in the negative control cells, while stimulation of the cells with PMA or PMA plus ionomycin resulted in significant increases of CD69 and CD25 expressions (74% and 64.1% increases, respectively) on the activated T cells. Pre-treating T lymphocytes with PAB at 10 μM significantly inhibited the expression of CD25 induced by co-stimulation of PMA plus ionomycin (Fig. 2A). Meanwhile, pre-treatment of PAB at the same concentration slightly suppressed the increased expression of CD69 on the T lymphocytes evoked by stimulation of PMA (Fig. 2B). This result suggests that the anti-proliferative effect of PAB is mainly correlated with the inhibition of

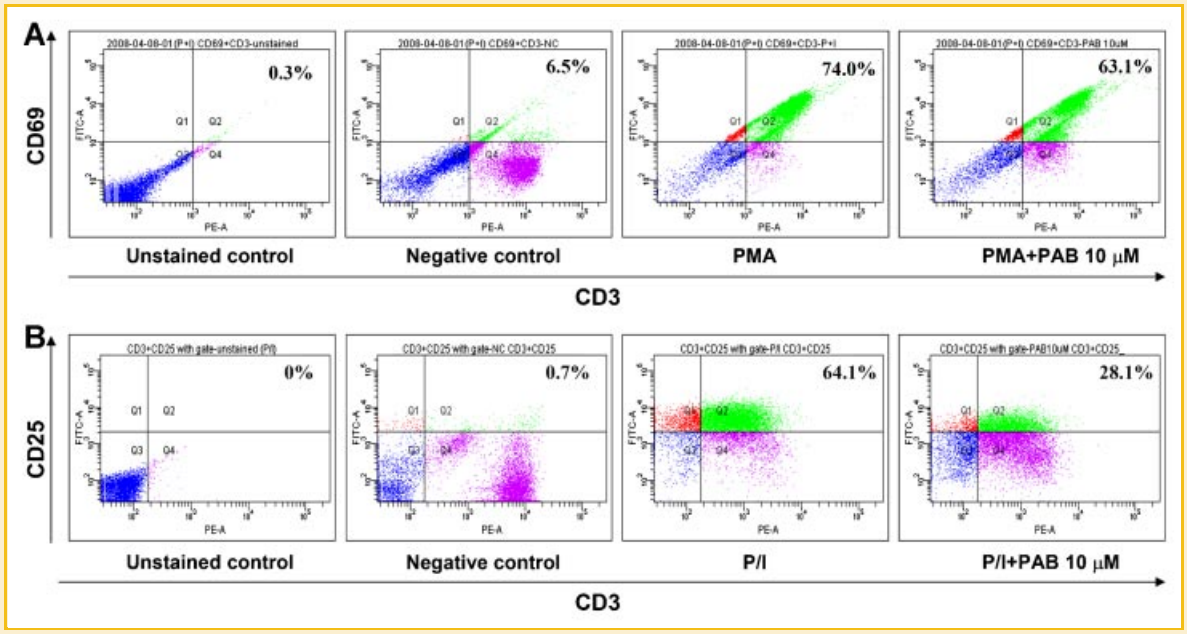

Fig. 2. Influences of PAB on the CD25 and CD69 expression of human T lymphocytes. The cells (10^6/well) were pre-treated with PAB for 2 h and then stimulated with PMA (20 ng/ml) for 24 h (A) or with PMA (20 ng/ml) plus ionomycin (1 μM) for 48 h (B), respectively. The cells were double stained with anti-CD3-PE and anti-CD69-FITC (A), or anti-CD3-PE and anti-CD25-FITC (B) antibodies and then analyzed by flow cytometry. The non-stimulated cells served as negative controls in the chart of flow cytometry analysis. Values represent percentages of double stained cells.

CD25 cell surface marker at the late stage of T lymphocyte activation.

PAB INHIBITS NUCLEAR TRANSLOCATION AND PHOSPHORYLATION OF NF-κB P65 INDUCED BY PMA PLUS IONOMYCIN

The inhibitory effect of PAB on cell proliferation and IL-2 secretion of the human T lymphocytes raises the possibility that PAB might affect NF-κB signaling, a crucial pathway involving T lymphocyte activation. It has been demonstrated that NF-κB involved p65 nuclear translocation [Gilmore and Herscovitch, 2006]. Thus, we determined the effect of PAB on NF-κB p65 nuclear translocation. Firstly, to determine the optimal incubation time of stimulators for activating NF-κB p65 nuclear translocation in human T lymphocytes, we performed a time kinetic study on NF-κB p65 nuclear translocation evoked by stimulation of PMA plus ionomycin. The results showed that the amount of p65 in the nuclei of the cells started to increase at 60 min after stimulation and reached maximum at 120 min (Fig. 3A, upper panel). These data suggest that incubation with PMA plus ionomycin for 120 min is appropriate to perform the experiment on NF-κB p65 nuclear translocation in human T lymphocytes. Accordingly, to determine whether the mechanism of the anti-proliferative effect of PAB on human T lymphocytes is in association with the NF-κB signaling pathway, cells were incubated with or without PAB at the indicated concentrations for 60 min and then stimulated with PMA plus ionomycin for 120 min. The results showed that p65 nuclear translocation was markedly increased in the activated T lymphocytes compared to the non-activated cells, while p65 nuclear translocation in PAB-pretreated cells was dose-dependently suppressed at drug concentrations of 1 to 10 μM (Fig. 3A, lower panel). This result strongly indicates that the suppression of p65 nuclear translocation in NF-κB signaling pathway is involved in the inhibitory effect of PAB on cell activation and proliferation of human T lymphocytes.

PAB INHIBITS IκBα PHOSPHORYLATION AND DEGRADATION INDUCED BY PMA PLUS IONOMYCIN

In the resting T lymphocytes, an inactive status of NF-κB transcriptional factor complex is retained by IκBα in the cytoplasm. Therefore, the inhibitory effect of PAB on NF-κB p65 nuclear translocation might also be related to the suppression of IκBα phosphorylation and degradation. In other words, it is valuable to examine if PAB could influence the upstream signaling of NF-κB. For this purpose, the effect of PAB on phosphorylation and degradation of IκBα of the human T lymphocytes activated by PMA plus ionomycin was investigated. The results clearly showed that PMA plus ionomycin could induce phosphorylation and degradation of IκBα of the cells in a time-dependent manner, and IκBα was almost completely degraded at 60 min after stimulation (Fig. 3B, upper panel). Accordingly, we made use of PMA plus ionomycin to activate the cells for 60 min to examine the effect of PAB on IκBα phosphorylation and degradation. The results showed that pre-treatment of PAB at concentrations of 1 to 10 μM resulted in a dose-dependent suppression on the IκBα phosphorylation and degradation of the human T lymphocytes (Fig. 3B, lower panel).

PAB SUPPRESSES P38 PHOSPHORYLATION IN THE MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY

Apart from NF-κB signaling, the mitogen-activated protein kinases (MAPKs) family, composed of ERK, JNK, and p38 kinase, is also

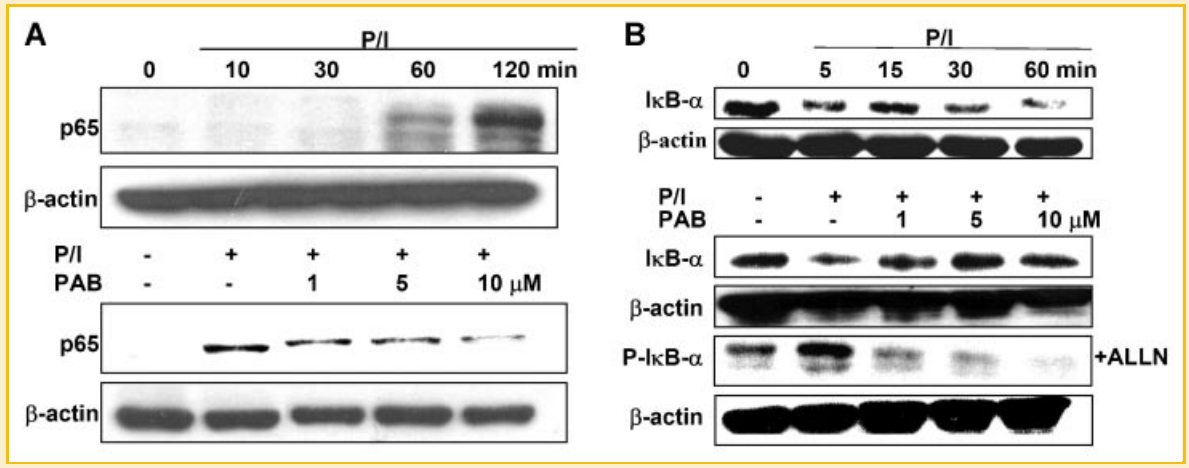

Fig. 3. Effects of PAB on p65 nuclear translocation and IκBα degradation and phorsphorylation of human T lymphocytes. A: Time kinetics of p65 expression and effect of PAB on p65 nuclear translocation of the cells. The cells were stimulated with 20 ng/ml PMA plus 1 μM ionomycin for the indicated time intervals (upper panel) or the cells were pre-treated with indicated concentrations of PAB for 60 min and then stimulated with 20 ng/ml PMA plus 1 μM ionomycin for 120 min (lower panel). B: Time kinetics of IκBα degradation and effects of PAB on IκBα degradation and phosphorylation of the cells. The cells were stimulated with 20 ng/ml PMA plus 1 μM ionomycin for the indicated time intervals (upper panel) or pre-treated with the indicated concentrations of PAB for 60 min and then stimulated with 20 ng/ml PMA plus 1 μM ionomycin for 60 min (lower panel). In the case of detecting P-IκBα, the cells were firstly pre-incubated with 100 μM ALLN and PAB for 60 min. The nuclear proteins or whole cellular proteins were prepared and then analyzed by Western blotting using antibodies against p65, IκBα, and P-IκBα. Similar results were obtained from two independent experiments.

responsible for the signal transduction during T lymphocyte activation. To investigate whether MAPKs are involved in PAB-mediated suppression of T lymphocyte activation, the time kinetics of MAPKs activation was studied in the cells stimulated with PMA plus ionomycin during different time intervals from 10 to 120 min. The results showed that the phosphorylations of ERK, JNK, and p38 kinase were significantly increased when the cells were incubated with PMA plus ionomycin for 10 min (upper panel of Fig. 4A–C). The phosphorylation forms of both JNK and p38 kinase reached their maximum expression at 10 min and then declined (upper panel of Fig. 4A,C). However, the phosphorylation form of ERK reached its maximum at 10 min and sustained this maximum for 60 min (Fig. 4B, upper panel). Therefore, 10 min incubation with PMA plus ionomycin was selected as the optimal time to stimulate the cells for

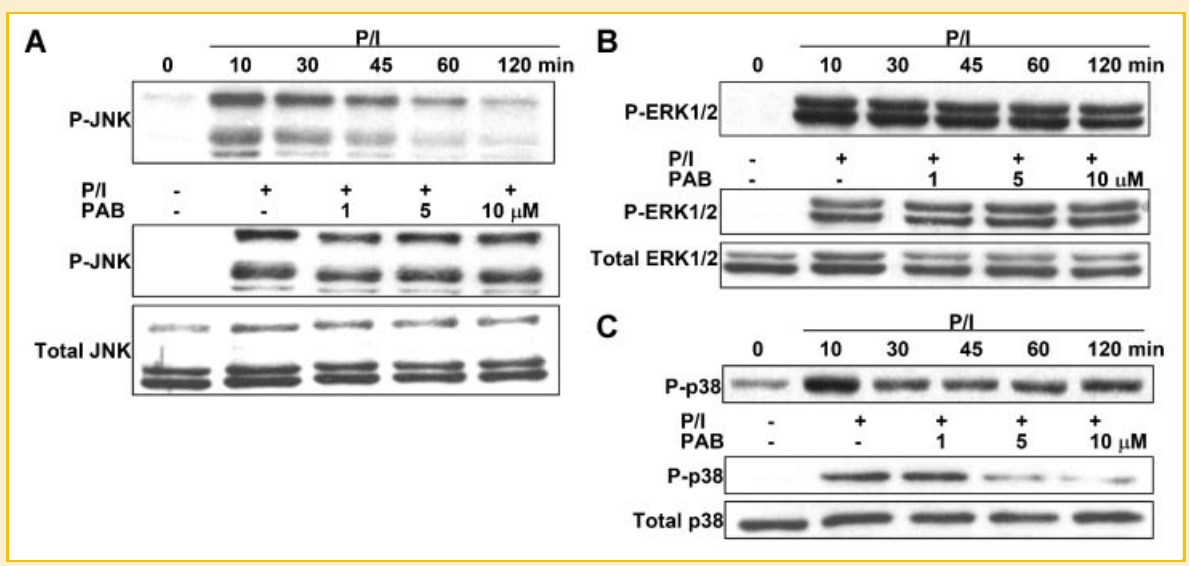

Fig. 4. Effect of PAB on mitogen-activated protein kinases pathway. A: The time kinetics of JNK phosphorylation (upper panel) and the effect of PAB (lower panel). B: The time kinetics of ERK1/2 phosphorylation (upper panel) and the effect of PAB (lower panel). C: The time kinetics of p38 kinase phosphorylation (upper panel) and the effect of PAB (lower panel). Cells were stimulated with 20 ng/ml PMA plus 1 μM ionomycin for the indicated time intervals in the time kinetic experiments of phosphorylation of JNK, ERK1/2, and p38. While for examination of the effects of PAB on the above MAPK kinases, the cells were pre-treated with indicated concentrations of PAB for 60 min and then treated with 20 ng/ml PMA plus 1 μM ionomycin for 10 min. After that, the phosphorylation forms and total forms of JNK, ERK1/2, and p38 kinase from cellular proteins were analyzed by Western blotting using antibodies against P-JNK, total JNK, P-ERK1/2, total ERK1/2, P-p38 kinase, and total p38 kinase.

analyzing ERK, JNK and p38 phosphorylation, so as to further elucidate the mechanisms by which PAB inhibits human T lymphocyte activation. In the subsequent experiments, the cells were pre-treated with PAB for 60 min and then stimulated with 20 ng/ml PMA plus 1 μM ionomycin for 10 min. The results demonstrated that PAB significantly and dose-dependently suppressed the activation of p38 kinase (Fig. 4C, lower panel), but failed to inhibit ERK and JNK phosphorylation in the PMA plus ionomycin-activated human T lymphocytes (lower panel of Fig. 4A,B). These results indicate that PAB can target p38 protein in the MAPKs pathway to suppress T cell activation and proliferation.

DISCUSSION

The root bark of *P. kaempferi* is commonly used in China as an antifungal and anti-inflammatory herbal remedy, and PAB has been reported to be a major chemical component of this medicinal herb [Wong et al., 2005; Wu et al., 2006]. Previous studies showed that PAB possesses potent pharmacological activities in inducing apoptosis in cancer cell lines [Wong et al., 2005] and anti-angiogenesis in human microvessel endothelial cells [Tong et al., 2006]. However, influences of PAB on the major immuno-component cells, the human T lymphocytes, remain unclear. In the current study, we examined for the first time the immunosuppressive effect of PAB on human peripheral T lymphocytes.

In the pathogenesis of autoimmune and inflammatory diseases, the central events of immune responses are the activation and clonal expansion of T cells. Optimal T cell activation requires two signals, one provided by the antigen-specific T cell receptor (TCR) complex and another provided by co-stimulatory receptor CD28 [Zhang et al., 1999; Diehn et al., 2002]. Co-stimulation of PMA plus ionomycin is usually applied to mimic TCR-CD3/CD28-mediated signaling in the process of T cell activation [Weng et al., 1996]. We thus examined the suppressive effect of PAB on the proliferation in human T lymphocytes stimulated by PMA plus ionomycin. The results showed that PAB significantly suppressed T lymphocytes proliferation. Meanwhile, it is well known that the antibodies against CD3 and CD28 are endogenous stimulators responsible for the body's immune responses through activation of CD3 lymphocytes, and thus we used anti-OKT3 plus anti-CD28 antibodies to activate the cells. Similarly, the results showed that PAB could also significantly suppress the cell proliferation evoked by the co-stimulation of anti-OKT3 plus anti-CD28 antibodies. The activation of T lymphocytes depends on the production of IL-2 that serves as a growth factor for T cells [Krummel and Allison, 1996; Pae et al., 2004]. Besides, IL-2 also plays a crucial role in the progression of antigen-activated T cells from G1 to S/G2/M phase of the cell cycle [Gately et al., 1991; Nguyen et al., 2000]. Our results demonstrated that PAB obviously inhibited IL-2 production when T lymphocytes were stimulated either by the co-stimulations of PMA plus ionomycin or anti-CD3 plus anti-CD28. These results strongly suggest that PAB may contribute to the suppression of the cell cycle and then to the inhibition of inflammatory conditions in the body.

Furthermore, MAPKs, including ERK, JNK, and p38 kinase, serve as the most ancient signal transduction pathways involved in T cell activation [Yoshizawa et al., 2008], as well as in autoimmune and inflammatory diseases [Schett et al., 2000; O'Neill, 2006]. All MAPK cascades play important roles in regulating IL-2 expression [Dong et al., 2002]. IL-2 expression can be up-regulated when ERK is activated, while the opposite response is observed when ERK activity is inhibited [Koike et al., 2003]. JNK could phosphorylate c-jun, a member of the AP-1 transcriptional factor family which has been shown to be induced upon T cell activation and involved in IL-2 gene transcriptional activity [Li-Weber et al., 2005; Wong et al., 2009]. Inhibition of p38 kinase can prevent the expression of IL-2, which means p38 kinase is essential for T-cell activation [Zhang et al., 1999]. It has also been reported that p38 kinase promotes nuclear translocation of the nuclear factor of activated T cells c (NFATc), one of the major transcription factors binding to IL-2 gene promoters, by activating NFATc promoter, stabilizing NFATc mRNA, increasing NFATc translation, and promoting NFATc-CREB-binding protein (CBP) binding [Wu et al., 2003]. Meanwhile, the inhibitor of p38 kinase, SB203580, blocks IL-2 production in both immature (thymocytes) and mature (splenic) T cells through inhibiting NFATc [Wu et al., 2003]. In the current study, we demonstrated that PAB significantly inhibited p38 phosphorylation while it failed to suppress ERK and JNK activities. Based on these results, we suggest that the suppressive effect of PAB on IL-2 expression and T lymphocyte activation might result from preventing p38 kinase activation.

To further understand the underlying mechanism of the suppressive action of PAB on T cell activation, we investigated the effect of PAB on T lymphocyte activation markers, that is, CD25 (IL-2α receptor) and CD69. In general, CD25 is highly expressed during T lymphocyte activation [Lin and Leonard, 1997; Kim et al., 2006; Camargo et al., 2009], and it appears to be regulated at the transcriptional level by CD28 through NF-κB signaling which is mainly regulated by classical NF-κB p50–p65 complexes [Kane et al., 2002], while CD69 is the earliest T lymphocyte activation marker [Caruso et al., 1997]. Both of the two markers participate in T lymphocyte proliferation and levels of the markers correlate with the degree of immune responses. Our results showed that PAB clearly inhibited CD25 expression but slightly suppressed CD69 expression on the activated T cells. This implies that PAB might suppress T cell activation at a late stage rather than the earliest stage through inhibiting the NF-κB pathway. From the suppression of T lymphocytes proliferation, IL-2 production and T lymphocyte activation marker expression, we have provided evidence that PAB exerts an inhibitory effect on T lymphocyte activation.

In light of the significant role of NF-κB signaling in T cell activation and cytokines secretion [Ho et al., 2004], we hypothesized that PAB might inhibit NF-κB signaling activation of T lymphocytes. Activation of NF-κB signaling is mediated by IKKβ which in turn phosphorylates and degrades IκBα [Ghosh and Karin, 2002]. IκBα is an inhibitory protein that retains the NF-κB transcriptional factor complex in the cytosol of the resting T cells [Epinat and Gilmore, 1999]. In the current study, we found that PAB dose-dependently suppressed IκBα phosphorylation and degradation and NF-κB p65 nuclear translocation. These results suggest that PAB

might inhibit T lymphocyte activation via the suppression of the NF-κB pathway.

Based on the potent ability of PAB to inhibit T lymphocytes proliferation, IL-2 production, CD25 expression, NF-κB p65 activation and p38 kinase phosphorylation, we conclude that IκBα-NF-κB signaling pathways and p38 kinase phosphorylation are involved in PAB-mediated immunosuppression in human T lymphocytes. These findings suggest that PAB merits further study as a novel anti-inflammatory agent with a wide range of important applications.

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