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Suppression of the NF-κB signalling pathway by ergolide, sesquiterpene lactone, in HeLa cells

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Abstract

We have previously reported that ergolide, a sesquiterpene lactone isolated from *Inula britannica*, suppresses inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression by inhibiting nuclear factor-κB (NF-κB) in RAW 264.7 macrophages. In this study, we show that ergolide suppresses the DNA binding activity of NF-κB and nuclear translocation of NF-κB p65 subunit, leading to the inhibition of NF-κB-dependent gene transcription in 12-O-tetradecanoylphorbol 13-acetate (TPA)-stimulated HeLa cells. We also show that ergolide decreases the degradation and phosphorylation of IκB, an inhibitory protein of NF-κB, and this effect is accompanied by a simultaneous reduction of IκB kinase (IKK) activity. However, ergolide does not inhibit in-vitro IKK activity directly, suggesting the possible involvement of upstream IKK kinases in the regulation of NF-κB activation. Furthermore, ergolide-mediated protein kinase Cα (PKCα) inhibition is involved in reduction of NF-κB inhibition, as demonstrated by the observation that dominant negative PKCα, but not p44/42 MAPK and p38 MAPK, inhibits TPA-stimulated reporter gene expression. Taken together, our results suggest that ergolide suppresses NF-κB activation through the inhibition of PKCα-IKK activity, providing insight for PKCα as a molecular target for anti-inflammatory drugs.

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Introduction

Sesquiterpene lactones are the active constituents of many medicinal plants from the Asteraceae family. Preparations from these plants are used in traditional medicine for the treatment of inflammation (Garcia-Pineres et al 2004). Numerous studies have established that they exert anti-inflammatory activity by regulating nuclear factor-kappa B (NF-κB) (Lyss et al 1997; Hehner et al 1998; Rungeler et al 1999; Mazor et al 2000). NF-κB has been reported to play an important role in immune and inflammatory responses through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors and inducible enzymes such as cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) (Vane et al 1994; Karin & Ben-Neriah 2000; Tak & Firestein 2001). Therefore, NF-κB activation has been considered to be a molecular target for the screening of anti-inflammatory activity of lead compounds.

The activation of NF-κB is regulated by the inhibitory proteins of the IκB family, such as IκBα, IκBβ and IκBε, which retain NF-κB in the cytoplasm. The phosphorylation, ubiquitination, and degradation of IκB, and subsequent dissociation of NF-κB from IκB mediate NF-κB activation (Hatada et al 2000; Je et al 2004). IκB kinases, such as IKKα and IKKβ, have been shown to phosphorylate specific amino-terminal serine residues of IκB proteins (Karin 1999; Karin & Delhase 2000). In addition, IKKα and IKKβ are also phosphorylated and activated by one or more upstream activating kinases. Among the upstream kinases, NF-κB inducing kinase (NIK) has been important for the regulation of IKK activity (Woronicz et al 1997). IKK-mediated IκB phosphorylation mediates the nuclear translocation of NF-κB, leading to activation of target genes in the nucleus (Delhase et al 1999).

We previously reported that ergolide, a sesquiterpene lactone from *Inula britannica*, inhibits iNOS and COX-2 expression in lipopolysaccharide (LPS)/interferon-γ-stimulated RAW 264.7 cells through the inhibition of NF-κB nuclear translocation (Whan Han et al

2001). Here, we further investigate the involvement of upstream kinases in mediating the ability of ergolide to block NF-κB signalling pathway. We show that ergolide inhibits 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated DNA-binding activity and nuclear translocation of NF-κB. We also show that ergolide decreases TPA-induced degradation and phosphorylation of IκB through the inhibition of PKCα activity, as demonstrated by the observation that overexpression of dominant negative protein kinase Cα (PKCα) fails to induce transcriptional activation of NF-κB in response to TPA, suggesting that PKCα might be the molecular target for ergolide-mediated suppression of NF-κB signalling pathway.

Materials and Methods

Reagents, plasmid constructs and antibodies

Ergolide was isolated from dried flowers of *Inula britannica* L. var *chinensis* Regel and structural identity was determined spectroscopically (^{1}H and ^{13}C NMR, IR, MS) as described previously (Whan Han et al 2001). TPA was purchased from Calbiochem (Darmstadt, Germany). The GFP-p65 NF-κB expression vector was kindly provided by Dr Warner C. Greene (Gladstone Institute of Virology and Immunology, University of California, San Francisco) (Chen et al 2001). Expression vectors for constitutively active PKC-α (326–672) (PKC-α CA) and dominant negative PKC-α (K368R) (PKC-α DN) were supplied by Dr Jae-Won Soh (Laboratory of Signal Transduction, Department of Chemistry, Inha University, Incheon, Korea). p65 NF-κB and IKKβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against IκBα, phospho-IκBα, phospho-IKKβ, phospho-p44/42 MAPK and phospho-p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture and cell stimulation

HeLa and 293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained at 37°C in humidified 5% CO_2–95% air in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cells were plated at a density of 60% in culture dishes, and then cultured in DMEM for one or two days before the experiments. For stimulation experiments, the culture medium was replaced with fresh DMEM containing 50 ng mL^{-1} TPA in the presence or absence of ergolide for the indicated times.

Electrophoretic mobility shift assay (EMSA) of NF-κB

Nuclear protein extracts were prepared for EMSA as described previously (Shin et al 2005). The sequences of probes are shown as follows (binding site is underlined). NF-κBU 5′-AGC-TTG-GGG-ACT-TTC-C-3′; NF-κBL 3′-C-CCC-TGA-AAG-*G*TC-GGG-5′.

Transfection of NF-κB-Luc reporter gene and luciferase assay

For NF-κB promoter analysis, HeLa cells were transfected with 1 μg of NF-κB-Luc reporter plasmid DNA using calcium phosphate precipitation methods. The luciferase activity of the cell lysates was measured according to the luciferase assay kit manual (Promega, Madison, WI) and normalized for the amount of the protein in cell lysates.

Fluorescence microscopy of NF-κB

For localization of p65, HeLa cells were transfected with GFP-p65 for 24 h and pre-treated with ergolide for 1 h, followed by TPA stimulation for 1 h. Samples were viewed using a fluorescence microscope (Olympus IX70).

Immunoprecipitation and IKK assay

Cells were pre-treated with or without ergolide for 1 h before TPA (50 ng mL^{-1}) stimulation for the indicated times. After treatment with TPA or ergolide (or both), cell lysates were prepared and clarified and the supernatants were subjected to immunoprecipitation and IKK assay as described previously (Yoon et al 2005).

Western blot analysis

Cell lysates were prepared, clarified and subjected to Western blot analysis as described (Shin et al 2005).

Statistical analysis

Statistical significance was evaluated using the Student's *t*-test or analysis of variance using the Kruskal–Wallis method. $P < 0.05$ was considered statistically significant.

Results and Discussion

We previously reported that ergolide inhibits iNOS and COX-2 expression in RAW 264.7 macrophages through the inactivation of NF-κB (Whan Han et al 2001). To further elucidate molecular mechanisms by which ergolide regulates TPA-induced NF-κB activation in HeLa cells, we first analysed the DNA-binding activity of NF-κB in response to ergolide treatment. Treatment of HeLa cells with TPA markedly stimulated the binding activity of NF-κB, compared with untreated cells; however, ergolide pre-treatment inhibited the binding of NF-κB in a dose-dependent manner (Figure 1A, upper panel). Sesquiterpene lactones have been known to alkylate the sulfhydryl groups of cysteine residues on NF-κB subunit p50 and p65 by a Michael-type addition reaction (Kumar et al 1992; Matthews et al 1992). Based on previous findings that DNA-binding activity of activated NF-κB can be directly modified by sesquiterpene lactones (Lyss et al 1998), we next examined whether ergolide can directly alter the DNA-binding activity of TPA-stimulated NF-κB in HeLa cells. Nuclear binding assay of NF-κB was performed in-vitro with or without ergolide in cell-free extracts prepared from

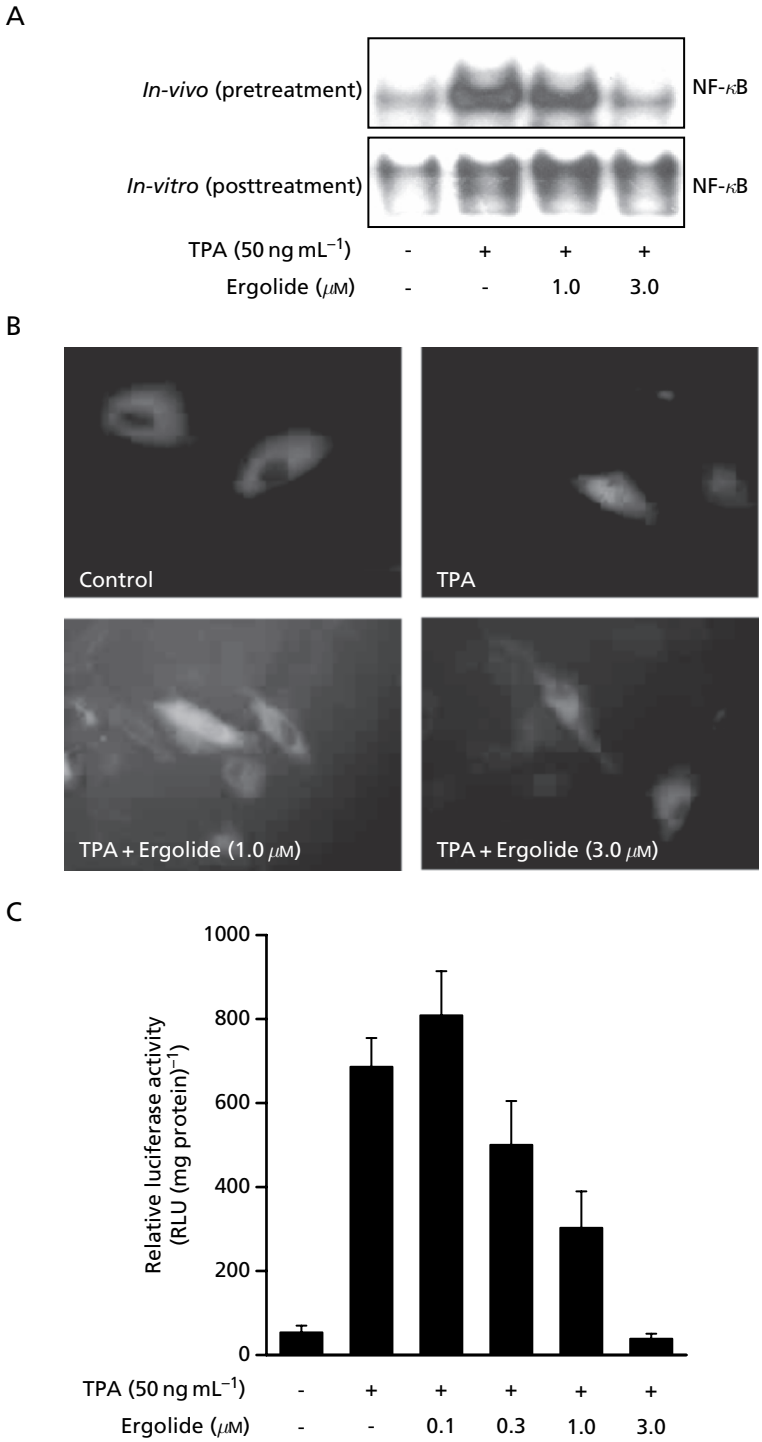

Figure 1 Effect of ergolide on DNA-binding activity, nuclear translocation of NF-κB and NF-κB promoter activity in TPA-stimulated HeLa cells. A. HeLa cells were plated at a density of 1.0×10^7 cells/100 mm culture dish in DMEM and incubated at 37°C. In the upper panel (in-vivo), after pre-treatment with either 1.0 or 3.0 μM ergolide for 1 h, the cells were stimulated with TPA (50 ng mL^{-1}) for 1 h. In the lower panel (in-vitro), cells were stimulated for 30 min with TPA (50 ng mL^{-1}) and nuclear extracts from the stimulated cells were pooled. These extracts were incubated for 30 min with 1.0 or 3.0 μM ergolide. The nuclear extracts were subjected to EMSA as described in Materials and Methods. B. HeLa cells were transfected with 1 μg of the GFP-p65 NF-κB expression vector for 24 h. After pre-treatment with 1.0 or 3.0 μM ergolide for 1 h, cells were stimulated with TPA (50 ng mL^{-1}) for 1 h. Subcellular localization of GFP-p65 were measured using fluorescence microscope as described in Materials and Methods. C. HeLa cells were transfected with 1 μg of NF-κB-Luc reporter plasmid DNA. Cells were pre-treated with 0.1–3.0 μM ergolide for 1 h and further incubated with TPA (50 ng mL^{-1}) for 8 h. Luciferase activity was determined and normalized to the protein content of each extract. Results from three independent transfections are presented as relative luciferase unit (RLU) per mg protein.

HeLa cells that were stimulated with TPA for 30 min. Unlike the inhibitory effect of ergolide on NF-κB activation in-vivo, ergolide post-treatment did not alter the DNA-binding activity of activated NF-κB in-vitro (Figure 1A, lower panel). These observations indicate that ergolide does not directly modify the active NF-κB and possibly regulates upstream target(s) of NF-κB signalling pathways.

Next, we performed fluorescence analysis to determine whether the translocation of the NF-κB subunit p65 is associated with ergolide-mediated inhibition of NF-κB DNA-binding activity. Upon transfection with the GFP-p65 NF-κB expression vector, HeLa cells were pre-incubated with ergolide (1.0 and 3.0 μM) for 1 h and then treated with 50 ng mL^{-1} of TPA for 1 h at 37°C. As shown in Figure 1B, while TPA caused nuclear translocation of the NF-κB subunit p65, ergolide treatment inhibited TPA-induced accumulation of NF-κB subunit p65 in the nucleus, indicating that the inhibition of NF-κB nuclear translocation is responsible for the inhibitory effect of ergolide on NF-κB DNA-binding activity. Furthermore, ergolide treatment abrogated the TPA-induced NF-κB transcriptional activation in a dose-dependent manner (Figure 1C). Collectively, these findings indicate that ergolide suppresses nuclear translocation and DNA-binding activity of NF-κB, leading to inhibition of NF-κB-dependent gene transcription.

To elucidate possible signalling mechanisms via which ergolide inhibits TPA-induced NF-κB activation, we examined the changes in degradation and phosphorylation of inhibitory protein of NF-κB (IκBα). TPA stimulation of HeLa cells decreased IκBα total protein levels below those observed in untreated cells (Figure 2A). In contrast, treatment with ergolide before TPA stimulation resulted in a significant increase in IκBα protein levels in a dose-dependent manner. We also examine whether the inhibitory effect of ergolide on TPA-induced IκBα degradation is modified by cysteine treatment. Addition of cysteine (1 mM) marginally attenuated ergolide inhibition of TPA-induced IκBα degradation (Figure 2A). IκB kinases (IKKs) are involved in the phosphorylation of IκB proteins, and the phosphorylation of IκB proteins on residues Ser-32 and Ser-36 facilitates protein turnover via a proteasome-dependent pathway. We further analysed the phosphorylation levels of total cellular IκBα using a specific anti-phospho-Ser32-IκBα antibody. As shown in Figure 2B, TPA markedly increased IκBα phosphorylation, but ergolide treatment before TPA stimulation decreased the phosphorylation levels of IκBα to basal levels, indicating that ergolide might modulate TPA-stimulated IKK activity, the upstream target of IκBα-NF-κB signalling pathways. Collectively, these findings suggest that ergolide-mediated suppression of NF-κB activation might be the result of the inhibition of IκBα degradation and subsequent NF-κB nuclear translocation rather than the direct modification of NF-κB, possibly through the inhibition of IKK activity.

To investigate if the regulation of IKK activity following ergolide treatment is involved in inhibition of IκBα phosphorylation and NF-κB activation, we performed IKK immunoprecipitation and IKK assay. TPA-stimulated IKK activity peaked within 15 min and gradually decreased until 1 h after treatment (data not shown). As anticipated, ergolide pre-treatment (3.0 μM) significantly decreased the TPA-stimulated IKK activity to basal levels (Figure 3A, upper panel). To determine

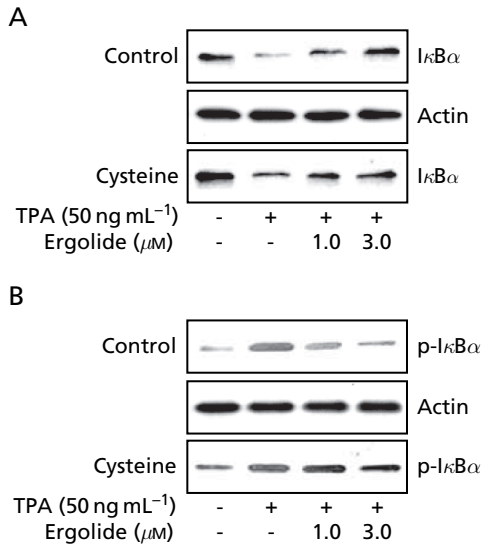

Figure 2 Ergolide inhibits TPA-induced degradation and phosphorylation of IκBα. HeLa cells were stimulated with TPA (50 ng mL^{-1}) for 45 min following pre-treatment with ergolide (1.0 or 3.0 μM) with or without cysteine (1 mM). Cellular extracts were Western-blotted with anti-IκBα (A) or anti-phopho-Ser32-IκB-α antibodies (B).

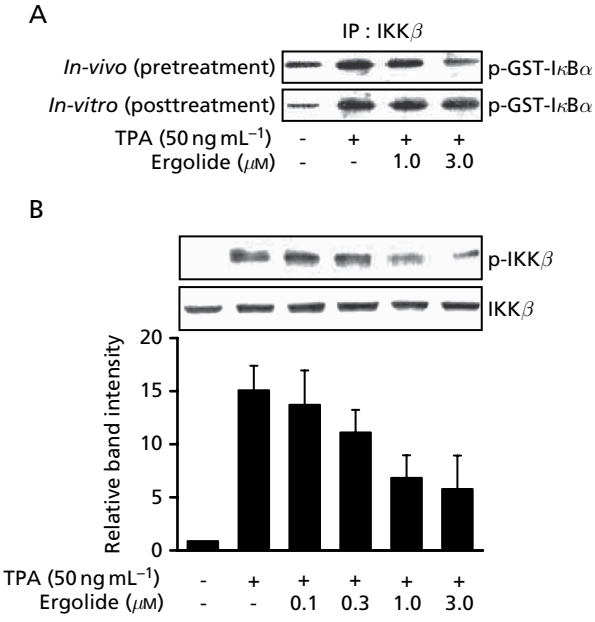

Figure 3 Effect of ergolide on IKK activity and phosphorylation of IKKβ in TPA-stimulated HeLa cells. A. In the upper panel, after pre-treatment with ergolide (1.0 or 3.0 μM) for 1 h, cells were stimulated with TPA (50 ng mL^{-1}) for 15 min. In the lower panel cells were stimulated with TPA for 15 min and the cytosolic fractions from the cell lysates were pooled and followed by ergolide treatment. The kinase activity of IKKβ immunoprecipitates was measured using GST-IκBα protein as substrate in the presence of [γ-^{32}P]ATP/ATP. B. After pre-treatment with ergolide (0.1–3.0 μM) for 1 h, cells were stimulated with TPA (50 ng mL^{-1}) for 15 min. Activity is expressed as fold increase relative to the untreated control.

whether the inhibitory effect of ergolide on IKK activity was due to direct interaction between ergolide and the IKK complex, we performed an in-vitro IKK assay. Ergolide did not inhibit TPA-stimulated IKK activity directly (Figure 3A, lower panel), suggesting the existence of upstream target(s) of IKK to be regulated by ergolide.

We next examined the effect of ergolide pre-treatment on the IKKβ phosphorylation status in HeLa cells. Following stimulation with TPA (50 ng mL^{-1}) for 15 min, IKKβ phosphorylation was determined by Western blot analysis in the presence or absence of ergolide. The inhibitory activity of ergolide pre-treatment for 1 h was concentration dependent for TPA-induced IKKβ phosphorylation (Figure 3B), demonstrating that ergolide inhibited the upstream kinase(s) that are involved in IKKβ phosphorylation. It has been reported that p38 MAPK and p44/42 MAPK play important roles in iNOS induction in LPS-stimulated mouse macrophages (Chen & Wang 1999; Lahti et al 2000). Some studies have also demonstrated that p38 MAPK activity is required for LPS-induced NF-κB activation (Chen et al 1999; Nick et al 1999). To examine the effect of ergolide on the activation of p44/42 MAPK and p38 MAPK in TPA-stimulated HeLa cells, we performed Western blot analysis with anti-phospho-specific antibodies. Pre-treatment with ergolide (3.0 μM) for 1 h before TPA stimulation (50 ng mL^{-1}) did not alter the phosphorylation of p44/42 MAPK or p38 MAPK, when compared with TPA-stimulated cells (Figure 4A). These observations indicate that ergolide might regulate IKKβ phosphorylation through another signalling pathway. Therefore, we investigated the functional role of PKCα in mediating the inhibitory effect of ergolide on NF-κB activation, as previous studies have demonstrated that PKCε and PKCα induce NF-κB-dependent reporter gene activation (Lozano et al 1994; Genot et al 1995; Trushin et al 1999), and TPA-induced IKK activation in U2OS cells is correlated with PKCα activation in TPA-stimulated COS-7 cells (Vertegaal et al 2000). As shown in Figure 4B, 293 cells treated with ergolide before TPA stimulation showed a significant reduction in NF-κB promoter activity, similar to that observed in HeLa cells. To examine the requirement for PKCα in ergolide-mediated inactivation of NF-κB, we analysed the effects of a dominant negative, catalytically inactive PKCα (PKCα DN) on ergolide-mediated NF-κB inactivation. The overexpression of PKCα DN in TPA-stimulated 293 cells inhibited NF-κB promoter activity to basal level, indicating that ergolide might regulate NF-κB activation through the inactivation of PKCα. In addition, the overexpression of a constitutively active PKCα (PKCα CA) in 293 cells increased NF-κB promoter activity up to 2-fold; however, this induction was abrogated by ergolide treatment (Figure 4C). Taken together, these findings demonstrate that ergolide suppresses the TPA-induced NF-κB transcriptional activation through the inhibition of PKCα activity, and suggest that PKCα might be one of the upstream kinases responsible for IKK phosphorylation in TPA-induced NF-κB signalling pathway.

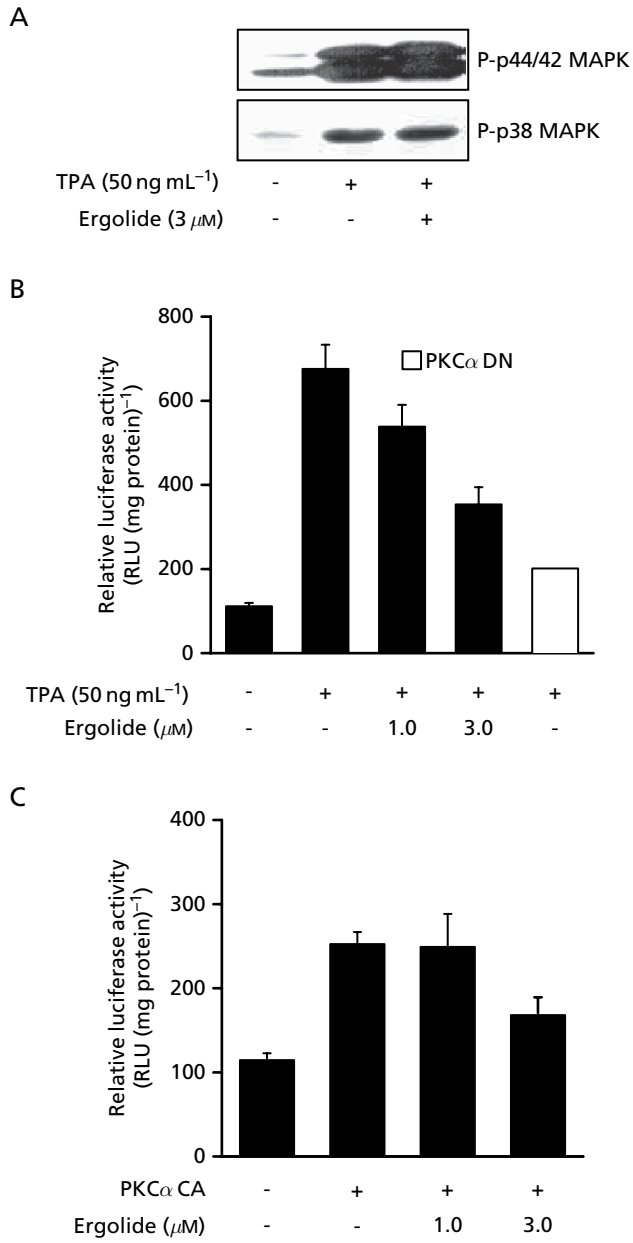

Figure 4 Involvement of PKCα in TPA-induced NF-κB activation in 293 cells. A. Following pre-treatment with ergolide for 1 h, the cells were stimulated with TPA (50 ng mL^{-1}) for 15 min. The phosphorylation levels of p44/42 MAPK and p38 MAPK were Western-blotted with antibodies. For NF-κB promoter analysis, 293 cells were cotransfected with 1 μg of NF-κB-Luc reporter plasmid DNA and an expression plasmid encoding dominant negative PKCα (PKCα DN) (B) or constitutively active PKCα (PKCα CA) (C). After transfection, the cells were pre-treated with ergolide for 1 h and then treated with TPA (50 ng mL^{-1}) for 24 h. Luciferase activity was determined and normalized to the protein content of each extract. Results from three independent transfections are presented as relative luciferase unit (RLU) per mg protein.

Conclusions

In this study we show that ergolide, a sesquiterpene lactone, suppresses NF-κB activation through the inhibition of PKCα, which is thought to be one of the upstream IKK kinases in NF-κB signalling pathway, leading to inhibition of TPA-stimulated IKKβ phosphorylation, IκB degradation, NF-κB nuclear translocation, NF-κB DNA-binding activity and transcriptional activation.

Collectively, our results demonstrate that ergolide inhibits NF-κB signalling pathway through the inactivation of PKCα-IKK activity. The identification of ergolide targets and further understanding of the signalling mechanisms could provide novel therapeutic strategies for the treatment of inflammatory diseases.

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