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Andrographolide interferes with binding of nuclear factor-κB to DNA in HL-60-derived neutrophilic cells

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1 Andrographolide, the major active component from *Andrographis paniculata*, has shown to possess anti-inflammatory activity. Andrographolide inhibits the expression of several proinflammatory proteins that exhibit a nuclear factor kappa B (NF-κB) binding site in their gene.

2 In the present study, we analyzed the effect of andrographolide on the activation of NF-κB induced by platelet-activating factor (PAF) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) in HL-60 cells differentiated to neutrophils.

3 PAF (100 nM) and fMLP (100 nM) induced activation of NF-κB as determined by degradation of inhibitory factor B α (IκBα) using Western blotting in cytosolic extracts and by binding to DNA using electrophoretic mobility shift assay (EMSA) in nuclear extracts.

4 Andrographolide (5 and 50 μM) inhibited the NF-κB-luciferase activity induced by PAF. However, andrographolide did not reduce phosphorylation of p38 MAPK or ERK1/2 and did not change IκBα degradation induced by PAF and fMLP.

5 Andrographolide reduced the DNA binding of NF-κB in whole cells and in nuclear extracts induced by PAF and fMLP.

6 Andrographolide reduced cyclooxygenase-2 (COX-2) expression induced by PAF and fMLP in HL-60/neutrophils.

7 It is concluded that andrographolide exerts its anti-inflammatory effects by inhibiting NF-κB binding to DNA, and thus reducing the expression of proinflammatory proteins, such as COX-2.
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Abbreviations: COX-2, cyclooxygenase-2; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; EMSA, electrophoretic mobility shift assay; ERK1/2, extracellular signal-regulated protein kinase; IκBα, inhibitory factor B α; iNOS, inducible nitric oxide synthetase; IKK, I-kappa kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappaB; PAF, platelet-activating factor

Introduction

Andrographolide is a labdane diterpene isolated from the leaves of *Andrographis paniculata*, a Chinese herbal medicine used for the treatment of viral infections and inflammatory diseases (Coon & Ernst, 2004). Andrographolide is known to exert several anti-inflammatory actions, including inhibition of intercellular adhesion molecule-1 expression in monocytes activated by tumor necrosis factor-α (Habtemariam, 1998), suppression of inducible nitric oxide synthetase (iNOS) expression in RAW264.7 cells stimulated by lipopolysaccharide (LPS) and interferon-γ (Chiou *et al*., 1998; 2000) and inhibition of microglial activation through inhibition of iNOS and cyclooxygenase-2 (COX-2) expression (Wang *et al*., 2004). In neutrophils, andrographolide reduces adhesion induced by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Shen *et al*., 2000), and reactive oxygen species production and Mac-1 expression induced by phorbol myristate acetate and fMLP (Shen *et al*., 2002).

Nuclear factor kappaB (NF-κB) is a transcription factor found in a great variety of immune cells participating in the regulation of genes involved in cellular and physiological process, such as growth and apoptosis, and has an important role in the inflammatory and immune response by inducing the transcription of proinflammatory genes (Baeuerle & Baltimore, 1996). For instance, the proinflammatory mediators such as intercellular adhesion molecule-1, iNOS and COX-2 are proteins regulated by the transcription factor NF-κB (Barnes & Karin, 1997; Roebuck & Finnegan, 1999). Activation of NF-κB involves the phosphorylation of specific inhibitory factors (IκBα, IκBβ and IκBε) by I-kappa kinase (IKK)1, IKK2 and NF-κB essential modulator (Karin & Delhase, 2000). Phosphorylated IκB is rapidly degraded in response to stimuli (Simeonidis *et al*., 1999). This degradation allows the resultant free NF-κB dimer to translocate into the nucleus and induces gene transcription (Baldwin, 1996;

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Ghosh *et al*., 1998). Activation of NF-κB has an important role in neutrophils, regulating apoptosis (Ward *et al*., 1999), production of interleukin-1, -6, -8 and adhesion molecules (Baeuerle & Henkel, 1994) and expression of COX-2 (Kim *et al*., 2001).

In neutrophils, NF-κB is activated by proinflammatory stimulus such as, fMLP and platelet-activating factor (PAF) (McDonald *et al*., 1997). fMLP and PAF are factors that mediate diverse neutrophil functions, such as chemotaxis, superoxide production and COX-2 expression. These factors bind to G-protein-coupled receptors, which induce mitogen-activated protein kinase (MAPK) activation (Chao & Olson, 1993; Ishii & Shimizu, 2000). PAF activates p38 and extracellular signal-regulated protein kinase (ERK)1/2 MAPK in neutrophils (Nick *et al*., 1997; Hidalgo *et al*., 2004; Khreiss *et al*., 2004). On the other hand, it has been demonstrated that fMLP activates mainly ERK1/2 protein in human neutrophils (Nick *et al*., 1997).

Several natural compounds have been reported to reduce NF-κB activation (Bremner & Heinrich, 2002) and in the present study, using neutrophil-like dimethylsulfoxide (DMSO) differentiated HL-60 cells, as a cellular model of neutrophils (Santos-beneit & Mollinedo, 2000), we have shown that NF-κB activation by PAF and fMLP is strongly inhibited by andrographolide, an effect that is mediated by andrographolide blocking the binding of NF-κB to DNA.

Methods

HL-60 cell culture and differentiation

The human leukemia promyelocytic cells (HL-60) were a kind gift from Dr Ricardo Hermosilla (Institute of Pharmacology, Charité-Universitätsmedizin Berlin, Berlin, Germany). The HL-60 cells were grown in RPMI 1640 medium, supplemented with 2 mM L-glutamine, penicillin–streptomycin and 10% fetal bovine serum, at 37°C under 5% CO_2. The HL-60 cells were differentiated by incubating them in RPMI 1640 medium plus 1.3% ($v\,v^{-1}$) of DMSO for 5 days, and the differentiation was monitored by nitro blue tetrazolium reduction. These differentiated cells will be referred to hereafter as HL-60/neutrophils.

Construction of reporter plasmid

The reporter plasmid pGL3-NF-κB was constructed by insertion of 5× NF-κB consensus oligonucleotide, containing in the ends the *Bgl*II and *Kpn*I restriction sites for cloning into the pGL3-promoter plasmid. *Escherichia coli* JM109 were transformed with the construct and plasmid DNA was isolated with the EZNA® Plasmid Midiprep Kit. Finally, the pGL3-NF-κB plasmid was sequenced.

Luciferase assay

The HL-60/neutrophils were transfected on the fourth day of differentiation, with 1 μg of pGL3-NF-κB plasmid and 0.5 μg of pRL-TK plasmid as control, in Fugene6 reagent for 24 h, according to the manufacturer's instructions. The cells were preincubated with andrographolide (5 and 50 μM) or vehicle for 30 min, and then stimulated with 100 nM PAF for 1 h. The cellular extracts were obtained and the luciferase activity was measured with the Dual-Luciferase Reporter Assay System in a luminometer (Lumat-Berthold, Germany). The luciferase activity was determined as the ratio of RLU pGL3-NF-κB/pRL-TK, and expressed as fold from the control.

Western blot

The HL-60/neutrophils (5×10^6 cells) were preincubated with andrographolide (50 μM) or vehicle for 15 min, and then incubated with PAF or fMLP (100 nM) for 2 min. For ERK1/2 and p38 phosphorylation analysis, total protein extracts were prepared, and 50 μg of proteins was analyzed by 12% SDS–PAGE. Immunoblotting was carried out with monoclonal anti-phospho-ERK1/2 and anti-ERK1/2, and anti-phospho-p38 and anti-p38, as described earlier (Hidalgo *et al*., 2004). The blots were developed with chemiluminescence system. The Scion Image from NIH software was employed to analyze the blots, and the phosphorylated proteins were normalized with the unphosphorylated proteins. For the analysis of COX-2 expression, 80 μg of protein was analyzed by 12% SDS–PAGE and immunoblotting was made with a monoclonal anti-COX-2 antibody. The blots were stripped and reprobed with anti-β-actin antibody.

Preparation of nuclear and cytosolic extracts

The HL-60/neutrophils (5×10^6 cells) incubated with andrographolide (10, 50 and 100 μM) or vehicle and then stimuled, were collected for the preparation of the cellular extracts, as described by Castro-Alcaraz *et al*. (2002). The protein concentration was measured by the method of Bradford, and the cytosolic and nuclear extracts were stored at −80°C. The cytosolic extract was used to analyze IκBα protein by Western blot (as described before), and the nuclear extracts were used for the DNA binding analysis.

Electrophoretic mobility shift assay (EMSA)

The NF-κB consensus oligonucleotide probe double strand (5′-AGTTGAGGGGACTTTCCCAGG-3′) was labeled with ^{32}P using T4 polynucleotide kinase and purified in BioSpin 30 Tris Columns. The binding reaction was in 25 μl, with 2 μg of nuclear extract, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 12.5% glycerol, 20 ng μl^{-1} poly dG-dC, 1 mM DTT, 0.1% Triton X-100 and 30,000 c.p.m. ^{32}P-probe, for 30 min on ice. For the competition assays, the binding reaction was performed in the presence of the unlabeled oligonucleotide (100-fold molar excess) and nonspecific unlabeled oligonucleotide (5′-AGTTGAGGCGACTTTCCCAGG-3′) (500-fold molar excess). The samples were analyzed by electrophoresis on 6% polyacrylamide native gels in 45 mM Tris-borate and, 1 mM EDTA, pH 8.0, and the gel was dried and exposed in a Molecular Dynamics Storm 860 Imager.

Cell viability

The cell toxicities of andrographolide on HL-60/neutrophils, at the concentrations and times described in our experiments, were assessed with Trypan blue exclusion and the CytoTox 96®, nonradioactive cytotoxicity assay (Promega, Madison, WI, U.S.A.).

Materials and reagents

PAF (C-16) and fMLP were obtained from Calbiochem (La Jolla, CA, U.S.A.). Andrographolide and monoclonal antibody to β-actin were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). pGL3 plasmid, pRL-TK plasmid and Dual-Luciferase Reporter Assay System were obtained from Promega (Madison, WI, U.S.A.). RPMI 1640 medium, penicillin, streptomycin, FBS and nitrocellulose membrane were purchased from Invitrogen (Grand Island, NY, U.S.A.). Monoclonal antibodies to phospho-ERK1/2, phospho-p38 and p38, and anti-rabbit IgG-peroxidase and anti-mouse IgG-HRP were obtained from New England Biolabs (Beverly, MA, U.S.A.). Polyclonal antibodies to ERK1 (sc-94) and IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal antibody to COX-2 was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). Protease inhibitors and Fugene6 reagent were obtained from Roche Diagnostics (Indianapolis, IN, U.S.A.). BioSpin 30 Tris Columns were obtained from Bio-Rad (U.S.A.). The EZNA® Plasmid Midiprep Kit was purchased from Omega Bio-Tek. (Doraville, GA, U.S.A.). Enhanced chemiluminescence reagent (ECL) was obtained from Amersham Biosciences (England). All other reagents and chemicals were purchased from Merck (Darmstadt, Germany). PAF was dissolved in ethanol at a stock concentration of 1 mM, and the aliquots were kept at −70°C, used for one experiment and then discarded.

Statistical analysis

The results were expressed as the fold of control increase and were shown as the mean±s.e.m. A one-way analysis of variance (ANOVA) was performed and a Dunnet's multiple comparison test was applied using GRAPH PAD V 2.0. Values of $P<0.05$ were taken to show a significant difference between means.

Results

Andrographolide inhibits NF-κB activation by PAF

In the present study, we first investigated the effect of andrographolide on NF-κB activation in HL-60/neutrophils by PAF, in an NF-κB reporter assay. The cells were co-transfected with the pGL3-NF-κB plasmid and pRL-TK plasmid on the fourth day of differentiation for 24 h, preincubated with andrographolide or vehicle and then stimulated with PAF. Andrographolide (5 and 50 μM) inhibited significantly the PAF-induced luciferase activity in the NF-κB reporter construct (Figure 1a and b), indicating that andrographolide interfered with NF-κB activation.

Effect of andrographolide on ERK1/2 and p38 MAPK phosphorylation in HL-60/neutrophils

PAF has been described as activator of p38 MAPK and fMLP as activator of ERK1/2 MAPK in human neutrophils (Nick *et al*., 1997). In HL-60/neutrophils, PAF was also able to induce phosphorylation of p38 and fMLP caused ERK1/2 phosphorylation at 2 min (Figure 2a). To assess the effects of

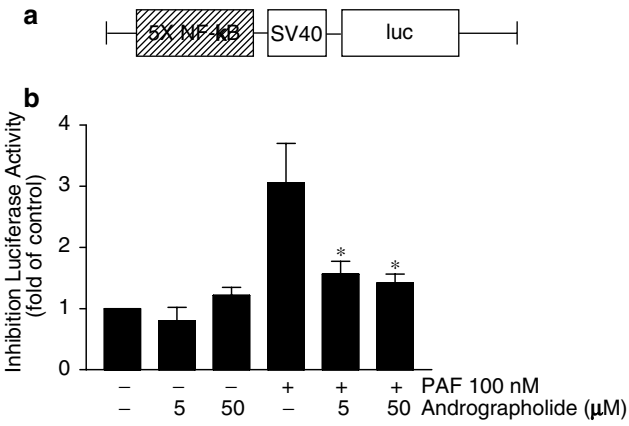

Figure 1 Inhibition of NF-κB-luciferase activity by andrographolide. (a) Schematic representation of NF-κB-luc construct. (b) HL-60/neutrophils were transiently transfected with the NF-κB-luc construct (Firefly luciferase) and a control gene (*Renilla* luciferase). The cells were preincubated with andrographolide (5 and 50 μM) for 30 min and stimulated with 100 nM PAF for 60 min. Luciferase activity was measured in a luminometer, and expressed as fold from control group; each bar represents mean±s.e.m., $n=3$. *$P<0.05$ vs PAF group.

andrographolide, the cells were preincubated for 15 min with andrographolide before they were stimulated with PAF or fMLP. Andrographolide (10 and 50 μM) did not inhibit the phosphorylation of either p38 MAPK or ERK1/2 (Figure 2a).

Effect of andrographolide on IκBα degradation in HL-60/neutrophils

Previously, it has been described that PAF and fMLP induce NF-κB activation in human neutrophils using a DNA binding assay (McDonald *et al*., 1997). Here, we measured the NF-κB activation as the IκBα degradation in the cytosolic extract using Western blot, and assessed if andrographolide had any effect on IκBα degradation. As expected, PAF and fMLP induced IκBα degradation at 30 min of stimulation in our cellular model. However, andrographolide (50 μM) did not inhibit IκBα degradation (Figure 2b).

Effect of andrographolide on DNA binding of NF-κB

To analyze the effect of andrographolide on DNA binding of NF-κB, we assessed the effect of andrographolide in whole cells and in nuclear extracts, using HL-60/neutrophils stimulated by PAF and fMLP, using the EMSA. PAF and fMLP induced DNA binding of NF-κB at 30 min of stimulation in HL-60/neutrophils. In whole cells preincubated with andrographolide (15 min) and then stimulated by PAF or fMLP, the DNA binding of NF-κB was inhibited by andrographolide (100 μM) (Figure 3). Andrographolide alone had no effect on DNA binding of NF-κB. To investigate the molecular target of andrographolide, we examined the effect of andrographolide on DNA binding of activated NF-κB in cell extracts. After stimulation of HL-60/neutrophils with PAF or fMLP for 30 min, nuclear extracts were prepared and then incubated with andrographolide. Andrographolide (10, 50 and 100 μM) inhibited DNA binding of activated NF-κB induced by PAF

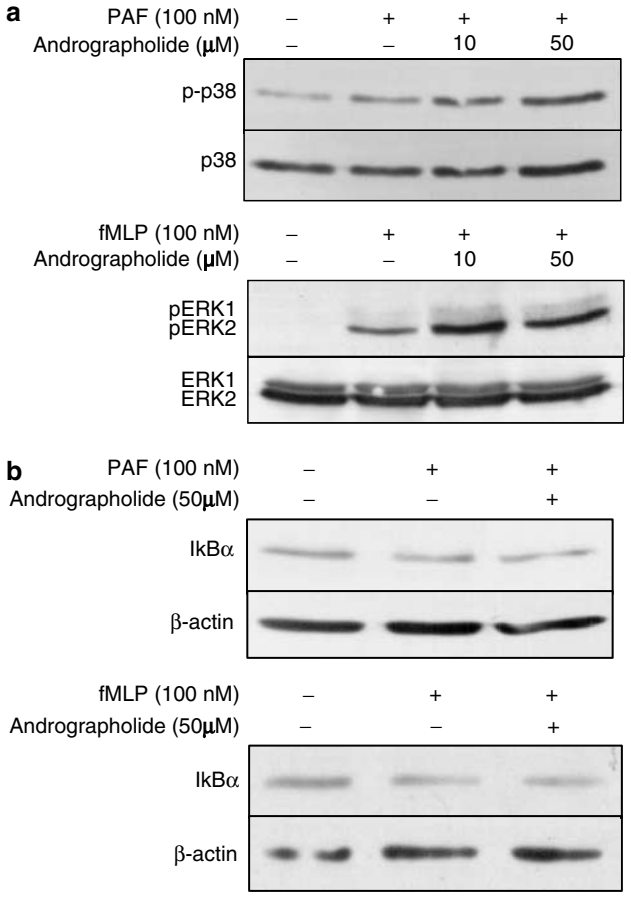

Figure 2 Andrographolide does not affect MAPK phosphorylation and IkBα degradation. (a) HL-60/neutrophils were pretreated with andrographolide (10 or 50 μM) or vehicle for 15 min, and then stimulated with PAF or fMLP (100 nM) for 2 min. Western blot analysis was made with p-ERK1/2, ERK1/2, p-p38 and p38 antibodies. (b) HL-60/neutrophils were pretreated with andrographolide (50 μM) or vehicle for 15 min, and then stimulated with PAF or fMLP (100 nM) for 30 min. IkBα was analyzed in the cytosolic extract by Western blot with anti-IkBα antibody. The amount of proteins was normalized with anti-β-actin antibody.

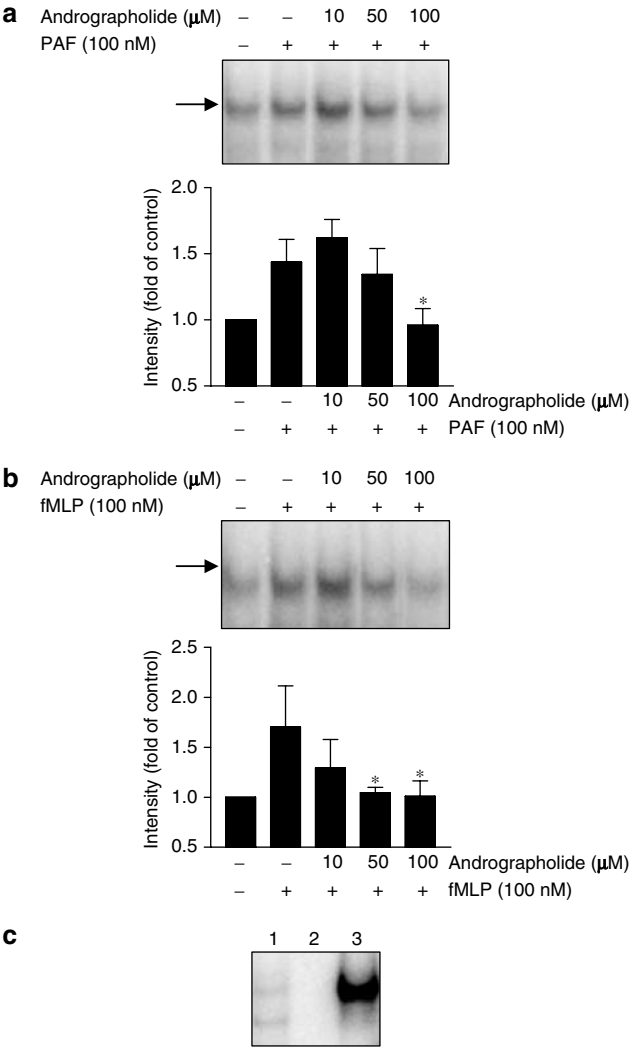

Figure 3 Andrographolide reduces DNA NF-κB binding *in vivo*. (a) HL-60/neutrophils stimulated with 100 nM PAF increased the DNA NF-κB binding and 100 μM andrographolide was able to inhibit this DNA binding. (b) fMLP (100 nM) increased the DNA binding of NF-κB and 50 and 100 μM andrographolide inhibited this effect. The arrow indicates specific binding of NF-κB. (c) EMSA assay. Lane 1, binding in the presence of 100-fold excess specific probe; lane 2, binding in the absence of nuclear extract; lane 3, binding in the presence of 500-fold excess mutated probe. For all experiments, 2 μg of nuclear proteins was assayed for the binding of DNA, resolved in 6% PAGE and analyzed (Methods). Each bar represents the mean ± s.e.m., n = at least 3. *$P<0.05$ vs PAF or fMLP group.

or fMLP (Figure 4), indicating that this compound directly interfered with the binding of NF-κB to DNA.

Effect of andrographolide on COX-2 expression

Expression of COX-2 is induced in human neutrophils by fMLP (Pouliot *et al*., 1998) and PAF induced COX-2 expression in a range of cell types (Bazan *et al*., 1997; Hermoso *et al*., 2001; Wang *et al*., 2002). Since COX-2 is regulated by NF-κB, we analyzed the effect of andrographolide on the expression of this protein. fMLP or PAF at a concentration of 100 nM induced the expression of COX-2 after 2 h of incubation in HL-60/neutrophils. Incubation with andrographolide (50 μM) inhibited the COX-2 expression induced by fMLP and PAF (Figure 5).

Discussion

Andrographolide has been shown to exhibit diverse anti-inflammatory activities (Visen *et al*., 1993; Chiou *et al*., 1998; Shen *et al*., 2002; Chen *et al*., 2004; Wang *et al*., 2004), but the underlying molecular mechanisms are still unknown. Here, we present evidence that andrographolide inhibits NF-κB action by directly interfering with its binding to DNA. Andrographolide prevents neither IκBα degradation nor MAPK phosphorylation following stimulation by PAF or fMLP.

Inhibition of the NF-κB activation by andrographolide seems to be at an intracellular level, and not at the level of the receptor, since andrographolide inhibited the NF-κB activation induced by fMLP and PAF, factors that bind to different G-protein-coupled receptors. Moreover, intracellular calcium mobilization induced by PAF and fMLP was not affected by andrographolide in the HL-60/neutrophils (data not shown).

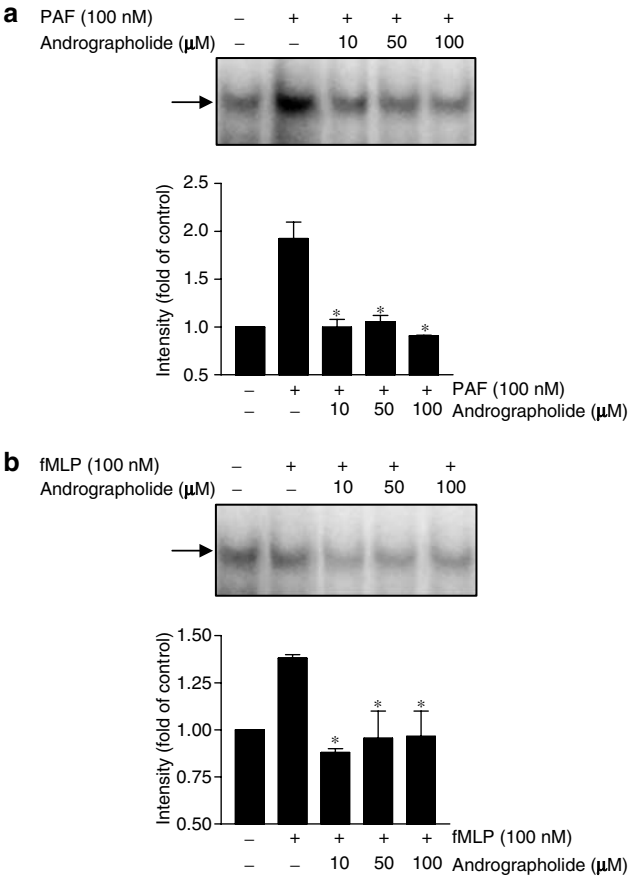

Figure 4 Andrographolide inhibition of DNA NF-κB binding *in vitro*. To analyze the effect of andrographolide *in vitro*, HL-60/neutrophils were stimulated with 100 nM PAF (a) or fMLP (b), and nuclear extracts were obtained as described in Methods. The nuclear extracts were incubated with andrographolide (10, 50 or 100 μM) for 15 min, and then the DNA binding reaction was carried out and analyzed as in Figure 3. The arrow indicates specific binding of NF-κB. Each bar represents the intensity fold of control, mean ± s.e.m., n = at least 3. $^*P<0.05$ vs PAF or fMLP group.

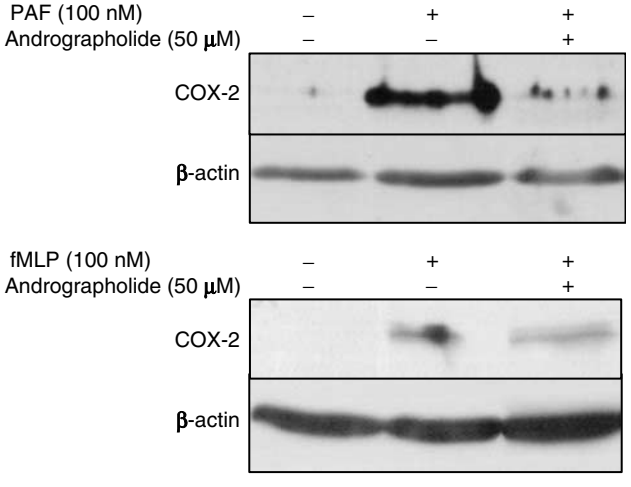

Figure 5 Andrographolide reduces COX-2 expression in HL-60/neutrophils. Cells were preincubated with 50 μM andrographolide for 15 min, and then stimulated with PAF or fMLP for 2 h. Total protein extracts were obtained and 80 μg of protein was used to analyze COX-2 expression and β-actin by Western blot with specific antibodies.

Similarly, other authors indicate that andrographolide did not reduce the calcium flux induced by fMLP in human neutrophils (Shen *et al*., 2002), and previously it has been demonstrated that andrographolide did not inhibit the binding of PAF to the cell membrane of bovine neutrophils (Burgos *et al*., 2004). Therefore, andrographolide seems to exert its antiinflammatory response downstream of the PAF or fMLP receptors. In support of this, andrographolide inhibited reactive oxygen species production in neutrophils stimulated either by fMLP or phorbol myristate acetate (Shen *et al*., 2002). Moreover, other responses induced by different stimuli can also be inhibited by andrographolide, that is, suppression of iNOS expression in RAW 264.7 cells stimulated by LPS/interferon-γ (Chiou *et al*., 2000). Recently, an attenuation of LPS-induced microglial activation and production of proinflammatory mediators such as nitric oxide, reactive oxygen species, tumor necrosis factor-α and prostaglandin E_2 by andrographolide has been described (Wang *et al*., 2004).

The p38 kinase and ERK1/2 are components of the intracellular MAPK signaling pathway activated by PAF and fMLP, and they play an important role in NF-κB activation. Thus, inhibition of p38 by SB203580 reduced NF-κB activation in neutrophils stimulated by LPS (Nick *et al*., 1999). In addition, inhibition of the ERK1/2 pathway by PD98059 completely prevented the activation of NF-κB induced by hydrogen peroxide (Jaramillo & Olivier, 2002). Therefore, we assessed the role of andrographolide on MAPK in our cell system. As expected, PAF or fMLP induced the MAPK phosphorylation in HL-60/neutrophils but andrographolide at doses that inhibited the activation of NF-κB did not modify the phosphorylation of these signaling kinases.

Our experiments of assessing degradation of IκBα and binding of NF-κB to DNA demonstrated that PAF and fMLP induced NF-κB activation in HL-60/neutrophils, as shown in human neutrophils (McDonald *et al*., 1997), indicating that HL-60 cells differentiated with DMSO provide a suitable model of neutrophils to study NF-κB activation.

Degradation of IκB is an essential step in NF-κB activation and follows on its phosphorylation by IKKs. Inhibition of IκB phosphorylation or of its degradation would lead to less NF-κB being available to bind to DNA. Andrographolide is a diterpene, and other natural diterpenes such as kaurane diterpene can inhibit IKK activity (Castrillo *et al*., 2001). However, we were not able to show any effects of andrographolide on IkB degradation. The final step in NF-κB activation is its binding to DNA and this was clearly inhibited by andrographolide, in either whole cells or in nuclear extracts. These findings are compatible with those of Lee *et al*. (2002), who showed similar inhibition with another natural diterpene.

As andrographolide interferes with a transcription factor, it should also affect the expression of target genes controlled by NF-κB during inflammatory processes. Expression of COX-2 is reduced by NF-κB inhibitors in endothelial cells stimulated by PAF (Marrache *et al*., 2002) and in microglial cells, andrographolide decreased COX-2 expression induced by LPS (Wang *et al*., 2004). Our results of decreased COX-2 expression after PAF or fMLP stimulation are in agreement with these previous findings and demonstrate further that the inhibition of NF-κB binding to DNA that we have shown, was able to decrease protein expression, as predicted.

Apoptosis in endothelial cells was suppressed by andrographolide (Chen *et al*., 2004) and NF-κB has been described to

regulate the cellular process of apoptosis, through the expression of antiapoptotic proteins. However, this effect of andrographolide on apoptosis was only attributed to an enhancement of PI3K–Akt activity, without the participation of NF-κB due to the absence of IkBα degradation (Chen *et al*., 2004).

However, other actions of andrographolide must also be considered. Thus, andrographolide has been demonstrated to inhibit the proliferation of cancer cells (Kumar *et al*., 2004). In leukemia cells (HL-60 cells), the inhibition of NF-κB by a mutant IκBα enhanced tumor necrosis factor-α-induced apoptosis (Cao *et al*., 2004). Recently, it has been demonstrated that andrographolide reduces T-cell activation and interferes with the experimental autoimmune encephalomyelitis in rodents (Iruretagoyena *et al*., 2005). These facts and the inhibition of NF-κB by andrographolide shown by us could explain the effect of andrographolide in breast cancer cells (Satyanarayana *et al*., 2004) and autoimmune encephalomyelitis (Hilliard *et al*., 1999).

During the editorial review of this paper, two other highly relevant papers were published. One showed inhibition of NF-κB binding to DNA by andrographolide, in a range of cell types (Xia *et al*., 2004). These authors showed a mechanism, similar to ours, for the anti-inflammatory actions of andrographolide, but with some minor differences. For instance, they used cell lines of transformed embryonic kidney (293 cells) and human umbilical vein endothelial cells stimulated by tumor necrosis factor-α in DNA binding assay. The other paper (Tsai *et al*., 2004) focused on signaling kinase pathways in RAW264.7 cells stimulated by C5a and found, as we did, that phosphorylation of p38 kinase was not affected by andrographolide. However, in contrast to our results, ERK1/2 phosphorylation was inhibited. This could indicate that other effects of andrographolide can contribute to the anti-inflammatory response.

In summary, the importance of NF-κB as a therapeutic target makes this transcription factor relevant to the development of new therapeutic strategies (Tak & Firestein, 2001; Ali & Mann, 2004; Karin *et al*., 2004). We conclude that andrographolide is an inhibitor of NF-κB binding to DNA, exhibiting strong potential anti-inflammatory properties and providing tools for cancer treatments and autoimmune diseases.

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