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RESEARCH PAPER

Kaurane diterpenes protect against apoptosis and inhibition of phagocytosis in activated macrophages

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Background and purpose: The kaurane diterpenes foliol and linearol are inhibitors of the activation of nuclear factor κB, a transcription factor involved in the inflammatory response. Effects of these diterpenes on apoptosis and phagocytosis have been analysed in cultured peritoneal macrophages and in the mouse macrophage cell line, RAW 264.7.
Experimental approach: Macrophages were maintained in culture and activated with pro-inflammatory stimuli in the absence or presence of diterpenes. Apoptosis and the phagocytosis in these cells under these conditions were determined.
Key results: Incubation of macrophages with a mixture of bacterial lipopolysaccharide (LPS)/interferon-γ (IFN-γ) induced apoptosis through a NO-dependent pathway, an effect significantly inhibited by foliol and linearol in the low μM range, without cytotoxic effects. Apoptosis in macrophages induced by NO donors was also inhibited. The diterpenes prevented apoptosis through a mechanism compatible with the inhibition of caspase-3 activation, release of cytochrome *c* to the cytosol and p53 overexpression, as well as an alteration in the levels of proteins of the Bcl-2 family, in particular, the levels of Bax. Cleavage of poly(ADP-ribose) polymerase, a well-established caspase substrate, was reduced by these diterpenes. Treatment of cells with foliol and linearol decreased phagocytosis of zymosan bioparticles by RAW 264.7 cells and to a greater extent by peritoneal macrophages.
Conclusions and implications: Both diterpenes protected macrophages from apoptosis and inhibited phagocytosis, resulting in a paradoxical control of macrophage function, as viability was prolonged but inflammatory and phagocytic functions were impaired.
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Keywords: foliol; linearol; macrophages; apoptosis; nitric oxide; lipopolysaccharide; interferon-γ; phagocytosis

Abbreviations: FITC, fluorescein isothicyanate; IFN-γ, interferon-γ; LPS, bacterial lipopolysaccharide; NOS-2, NO synthase-2; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; TMRM, tetramethylrhodamine methyl ester; TNF-α, tumor necrosis factor-α

Introduction

Kaurane diterpenes have been identified from numerous medicinal plants, which have been used for treatment of inflammation and cancer (de las Heras *et al*., 1999; Castrillo *et al*., 2001; Hwang *et al*., 2001; da Araujo *et al*., 2006). We have previously shown that the kaurene diterpenes, foliol and linearo,l selectively inhibit activation of nuclear factor κB (NF-κB), a central mediator of apoptosis and immune responses that regulates the expression by macrophages of a set of genes involved in host defense, such as pro-inflammatory cytokines, nitric oxide synthase-2 (NOS-2), cyclooxygenase 2 (COX-2), cell adhesion molecules and various matrix metalloproteinases. These two diterpenes also inhibit the expression of NOS-2 and COX-2 and the release of tumour necrosis factor-α (TNF-α) in macrophages challenged with bacterial lipopolysaccharide (LPS) (Castrillo *et al*., 2001).

In addition to the regulation of pro-inflammatory pathways, these kauranes might influence cell viability and apoptosis. Apoptosis is characterized by the ordered cleavage of cellular proteins and DNA, rendering the cells ready to be taken up by phagocytosis. Key to this ordered destruction is the activation of caspases (Shi, 2002; Lavrik *et al*., 2005). Two major routes, extrinsic and intrinsic, have been identified for the induction of apoptosis (Sprick and Walczak, 2004). The first pathway is mediated by death receptors, such as Fas or

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TNF receptors, and pro-caspase 8 is cleaved to the active form with subsequent activation of downstream caspases (caspase-3, -6 and -7). In the second pathway, mitochondria play essential roles through the mitochondrial permeability transition (PT). Induction of PT leads to dissipation of the inner transmembrane potential ($\Delta\psi_m$) and is one of the putative mechanisms triggering cytochrome *c* release to the cytosol and subsequently, caspase activation. The integrity of the mitochondrial membrane is regulated by proteins of the Bcl-2 family. Members of this family have both anti-apoptotic (Bcl-2 or Bcl-x_L) and pro-apoptotic activities (Bax, Bak) (Kroemer, 1997; Petit *et al*., 1997). Several pathways leading to apoptosis in macrophages have been characterized, involving NO-dependent and -independent mechanisms. Elevated NO synthesis after NOS-2 expression by activated macrophages is one of the main cytostatic, cytotoxic and pro-apoptotic mechanisms participating in the innate response (Hortelano *et al*., 1999). The pro-apoptotic activity of NO has been well documented and it involves the release of cytochrome *c* from the mitochondria (Hortelano *et al*., 1999, 2000, 2002), a rise in the tumour suppressor p53 (Brockhaus and Brune, 1999; Hortelano *et al*., 2000, 2002) and the accumulation and targeting of pro-apoptotic members of the Bcl-2 family, in particular, Bax (Sprick and Walczak, 2004).

In response to stimuli, macrophages also carry out phagocytosis, a key step in the process of resolution of inflammation (Aderem and Underhill, 1999; Aderem, 2003). In view of the ability of these diterpenes to inhibit macrophage-dependent NO synthesis, we have investigated the effect of foliol and linearol on macrophage viability (apoptosis) and phagocytosis, using both the macrophage cell line RAW 264.7 cells and elicited murine peritoneal macrophages in primary culture. Our results show that these diterpenes, in the range of the concentrations that exert a moderate inhibition of NOS-2 expression in LPS-activated macrophages, prevent apoptosis through a mechanism compatible with the inhibition of caspase-3 activation, p53 overexpression, as well as a change in the levels of proteins of the Bcl-2 family, favouring an anti-apoptotic pattern. In addition to this, these diterpenes impaired the phagocytosis of activated peritoneal macrophages, and to a lesser extent in RAW 264.7 cells.

Materials and methods

Isolation of peritoneal macrophages and cell culture conditions
Elicited peritoneal macrophages were obtained from male mice 4 days after intraperitoneal administration of 1 ml of 10% thioglycollate broth as described previously (Terenzi *et al*., 1995). Peritoneal macrophages and the murine macrophage cell line RAW 264.7 cells were seeded at densities of 2×10^6 cells in 6-cm plates or of 5×10^5 cells in 24-multiwell plates and cultured with RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin) at 37°C in an humidified atmosphere with 5% CO_2 and were used as described previously (Castrillo *et al*., 2001).

Flow cytometric analysis of apoptosis
Propidium iodide (PI) staining was performed after incubation of the cells with the appropriate stimuli in the presence of 0.005% PI, following a previous protocol (Hortelano *et al*., 2002). The quantification of the percentage of apoptotic cells was performed using a dot plot of the forward scatter against the PI fluorescence and the nature of the gated populations (viable, apoptotic and necrotic) was confirmed by cell sorting and TUNEL analysis with a commercial kit from Roche, as described previously (Genaro *et al*., 1995; Hortelano *et al*., 1997). In another set of experiments, after PI staining, cells were washed twice with phosphate-buffered saline (PBS) and then incubated with annexin V at room temperature, following the recommendations of the supplier (Annexin V-fluorescein isothicyanate (FITC) Kit, Bender Med System). Cells were scraped off the dishes, maintained at 37°C and immediately analysed in a Cyan MLE-R (DAKO-Cytomation) flow cytometer.

Flow cytometric analysis of the mitochondrial inner transmembrane potential
Cells were incubated for 30 min with the different diterpenes and then stimulated with LPS (250 ng ml^{-1})/interferon (IFN)-γ (40 U ml^{-1}) for 2 h. After these treatments, cells were incubated for 15 min at 37°C in the presence of the potential-sensitive probe tetramethylrhodamine methyl ester (TMRM) (0.05 μM) and Hoechst 33258 (5 μg ml^{-1}), followed by analysis in a Cyan MLE-R (DAKO-Cytomation), equipped with three excitation wavelengths (488, 635 and 365 nm). The fluorescence in the presence of 10 μM of the uncoupling agent m-chlorophenylhydrazone carbonyl cyanidine (mClCCP) was measured to define the maximal decrease of $\Delta\Psi$m. The fluorescence of TMRM in untreated cells was considered as 100% (Hortelano *et al*., 1997).

NO measurement
NO levels were determined spectrophotometrically by the accumulation of nitrite in the medium. Nitrite was determined with Griess reagent. The absorbance at 548 nm was compared with a standard of $NaNO_2$.

Preparation of cytosolic and total protein extracts
RAW 264.7 cells were washed twice with ice-cold buffer A (10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, 2 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 2 μg ml^{-1} *N*-tosyl-L-lysine-chloromethyl ketone, 5 mM NaF, 1 mM $NaVO_4$, 10 mM Na_2MoO_4) containing 120 mM NaCl and scraped off the plate. Cells were lysed at 4°C with 0.2 ml of buffer A supplemented with 0.5% Nonidet P-40, with continuous shaking. After centrifugation of the cell lysate the supernatant was stored at −80°C (cytosolic extract). The presence of cytochrome *c* in the cytosol was determined by western blotting cell extracts obtained by controlled lysis of the plasma membrane as described previously (Hortelano *et al*., 2002). Total cell extracts were prepared after homogenization of the cells with buffer A supplemented with 0.5%

CHAPS. Protein content was assayed using the Bio-Rad protein reagent. All steps of cell fractionation were carried out at 4°C.

Western blot analysis of proteins
Protein extracts were size-separated in 10–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The gels were blotted onto a Hybond-P membrane (GE Healthcare, Madrid, Spain) and incubated with the following antibodies (Abs): anti-p53, anti-Bax, anti-Bcl-2, anti-PARP (poly(ADP-ribose)polymerase) and anti-cytochrome *c* Ab (BD, San Jose, CA, USA). The blots were submitted to sequential re-probing with Abs after treatment with 100 mM β-mercaptoethanol and 2% SDS in Tris-buffered saline and heated at 60°C for 30 min. The blots were revealed by enhanced chemoluminiscence (ECL), following the manufacturer instructions (GE Healthcare, Amersham, UK).

In vivo *caspase assays*
In vivo activation of caspase-3 in apoptotic cells was measured by flow cytometry after labelling the cells with the caspase-3 inhibitor DEVD-FMK conjugated to FITC, following the instructions of the supplier of the CaspGLOW fluorescein active caspase-3 staining kit (MBL, Wobum, MA, USA).

Phagocytosis assay
For phagocytosis assays, macrophages (10^5 cells ml^{-1}) were placed in sterile 24-well tissue culture plates and cells were incubated with different concentrations of diterpenes for 30 min. Then, a suspension of zymosan-FITC fluorescent bioparticles (Invitrogen, Carlsbad, CA, USA) in culture medium, used as targets for macrophage phagocytosis, was added to the cells and phagocytosis allowed to proceed for 2 h at 37°C in a humidified 5% CO_2 incubator. The ratio of particles to cells was 10:1. Cells were washed with PBS and a mixture of medium plus PI was added. Fluorescence was determined by using a Beckman Coulter Cytomics FC 500. We have measured phagocytosed particles (positive fluorescence) and quantified differences of this fluorescence after treatment of cells with the diterpenes. The data were normalized to the number of living cells. Three controls were performed: cells treated with different concentrations of the diterpenes and without zymosan-bioparticles, cells only treated with zymosan-bioparticles and cells treated with zymosan-bioparticles plus a LPS/IFN-γ mixture. Phagocytosis was determined by washing off non-phagocytosed particles with PBS.

Statistical analysis
The data shown are the means ± s.d. ($n = 3$–4). Statistical significance was estimated with Student's *t*-test for unpaired observations. A value of $P < 0.05$ was considered significant. In studies of western blot analysis, a linear correlation between increasing amounts of input protein and signal intensity was observed (correlation coefficients higher than 0.84).

Chemicals
Foliol and linearol were obtained by purification as described by Castrillo *et al*. (2001). Other reagents were from Roche (Mannheim, Germany), Calbiochem (Darmstadt, Germany), Bachem (Bubendorf, Switzerland) and Sigma (St Louis, MO, USA). Fluorescent probes and antibodies were from BD (San Diego, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Culture media were from BioWhittaker (Verviers, Belgium).

Results

Foliol and linearol protect macrophages from LPS/IFN-γ-induced apoptosis
As Figure 1a shows, the levels of NOS-2 and the accumulation of NO in the culture medium of LPS/IFN-γ-activated RAW 264.7 cells and peritoneal macrophages were inhibited dose dependently by these diterpenes (RAW cells: 28 and 45% in the presence of foliol and linearol 50 μM, respectively; peritoneal macrophages: 37 and 45%, respectively). Incubation for 24 h of macrophages with LPS/IFN-γ-induced apoptotic death as deduced by the shift in the staining with PI. These diterpenes did not influence the viability of the cells as assessed by flow cytometry; however, these compounds exerted an important protection against the apoptosis triggered by LPS/IFN-γ. Doses of foliol and linearol in the 10–60 μM range protected the macrophages from apoptosis without cytotoxic effects (Figure 1b). This protection was more efficient in the case of peritoneal macrophages (50% inhibition at 40 μM) than in RAW 264.7 cells (40% inhibition, $n = 4$, $P < 0.05$). The anti-apoptotic effect of these diterpenes was also evaluated in the presence of 1400 W (a NOS-2 inhibitor, 10 μM) at different incubation times. Co-incubation of cells with diterpenes (50 μM) and 1400 W significantly decreased apoptosis triggered by LPS/IFN-γ in both types of cells (Figure 1c). However, this effect was not changed when 1400 W was added 8 h after challenge with LPS/IFN-γ.

Caspase activation is attenuated by foliol and linearol in cells treated with LPS/IFN-γ
The effects of the diterpenes on proteins involved in the apoptotic pathway were studied. The *in vivo* activation of caspase-3 was determined after loading the cells with a substrate-specific intracellular fluorescent probe. As Figure 2a shows, apoptosis was also attenuated significantly by foliol and linearol in both types of cells. To analyse more specifically the contribution of the NO-dependent pathway to apoptosis in macrophages treated with diterpenes, NO was generated with chemical NO donors. As Figure 2b shows, *S*-nitrosoglutathione (GSNO) induced a rapid apoptotic death in RAW 264.7 cells, as reflected by the increase of annexin V binding to the plasma membrane (6 h of incubation). Preincubation of cells with diterpenes for 30 min or 24 h reduced the annexin V binding when GSNO (100 μM) was used as the apoptotic stimulus. However, when the same experiment was performed using staurosporine (100 nM) to induce apoptosis, the protective effect by the

diterpenes was lost, suggesting that the anti-apoptotic effect is dependent mainly on the inhibition of NOS exerted by the diterpenes (Figure 2c). Similar results were obtained in peritoneal macrophages.

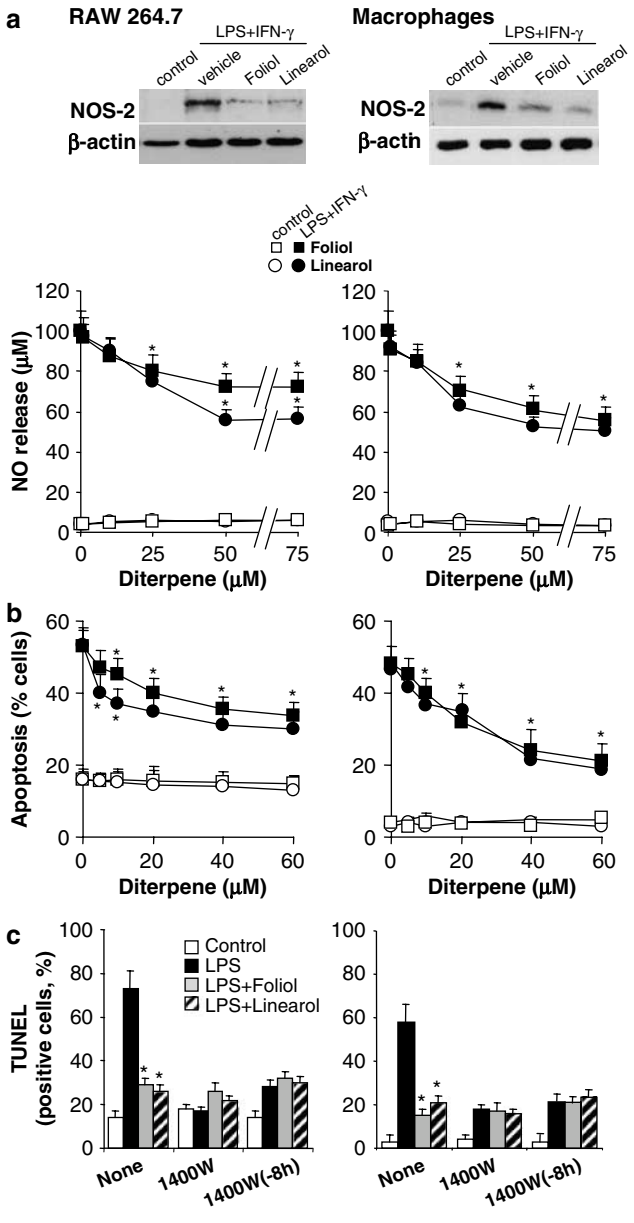

Figure 1 Foliol and linearol decreased NO synthesis and apoptosis of macrophages after treatment with LPS/IFN-γ. RAW 264.7 cells (left panels) and cultured peritoneal macrophages (right panels) were incubated with foliol and linearol for 30 min at the indicated concentrations and stimulated for 17–24 h with 250 ng ml^{-1} LPS and 40 U ml^{-1} IFN-γ. After 24 h of incubation, the accumulation of nitrite in the culture medium was measured with Griess reagent; *inset:* western blot corresponding to the NOS-2 levels at 24 h after treatment with 50 μM diterpene (**a**). The cells were analysed by flow cytometry after PI labelling and the percentage of apoptotic cells was determined after pre-incubation with different concentrations of diterpenes (**b**). Apoptosis was also determined by TUNEL in the presence of 1400 W (NOS-2 inhibitor, 10 μM) co-incubated with diterpenes or added 8 h after LPS/IFN-γ-stimulation (**c**). Results show the means $\pm$ s.d. of four experiments. *$P<0.01$ with respect to the LPS/IFN-γ condition. IFN, interferon; LPS, bacterial lipopolysaccharide; NOS-2, NO synthase-2; PI, propidium iodide.

As mitochondria plays a key role in the control of apoptosis, we also evaluated mitochondrial function in response to the diterpenes in terms of cytochrome *c* release and analysis of mitochondrial potential ($\Delta\Psi$m). NO-dependent apoptosis in macrophages involves a sustained $\Delta\Psi$m compatible with the release of mitochondrial mediators of apoptosis (Hortelano *et al*., 1999). After 8 h of LPS/IFN-γ challenge, a time at which a significant fall of $\Delta\Psi$m occurs, the potential remained stabilized in RAW 264.7 cells treated with the diterpenes (Figure 3a), at the time when the release of cytochrome *c* was attenuated under these conditions (Figure 3b). Moreover, when the levels of proteins that participate in the promotion of apoptotic death in macrophages were determined, the incubation of the cells with

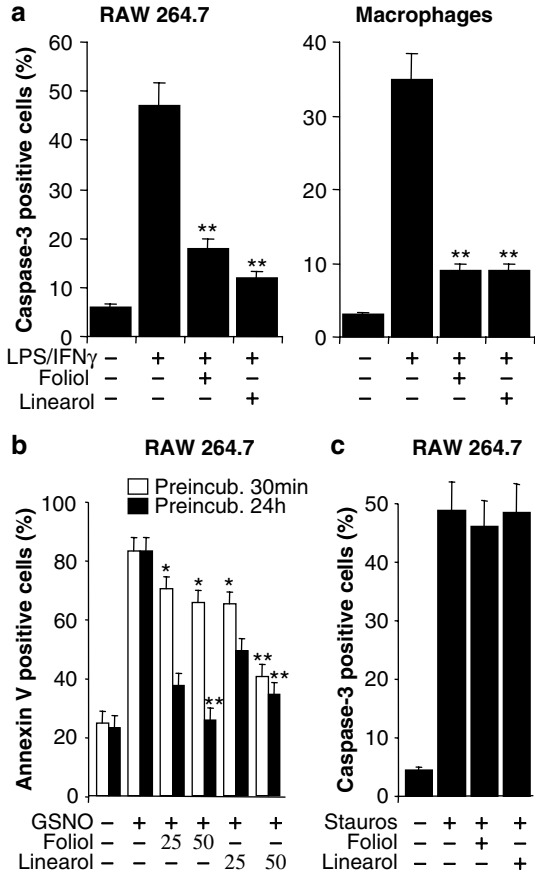

Figure 2 Inhibition by kaurane diterpenes of caspase 3 activation and apoptosis induced by low doses of GSNO. RAW 264.7 cells or peritoneal macrophages were treated with the diterpenes and LPS/IFN-γ as indicated in Figure 1. The intracellular activity of caspase-3 was determined after loading the cells with a fluorescent selective substrate (**a**). The percentage of annexin V-positive cells was determined by flow cytometry after preincubation (30 min or 24 h) of macrophages with the indicated concentrations of diterpenes followed by apoptosis induction by GSNO (100 μM) for 6 h (**b**). The intracellular activation of caspase-3 in response to staurosporine treatment (100 nM) was determined in the presence or absence of diterpenes and the percentage of positive cells was measured by flow cytometry (**c**). Results show the means $\pm$ s.d. of four experiments. *$P<0.05$, **$P<0.01$ vs the LPS/IFN-γ (**a**); vs GSNO (**b**). GSNO, *S*-nitrosoglutathione; IFN, interferon; LPS, bacterial lipopolysaccharide.

these diterpenes contributed to favour an anti-apoptotic profile. The amount of p53 and Bax decreased significantly, whereas the levels of Bcl-2 remained slightly higher vs cells challenged with LPS/IFN-γ (Figures 3b and c). In agreement with these observations, the integrity of PARP, used as a marker of *in vivo* caspase activation, confirmed the significant protection exerted by the diterpenes on activated cells, as indicated by the moderate appearance of the cleaved 32 kDa PARP fragment (Figure 3b).

Foliol and linearol reduced phagocytic activity of macrophages

Preincubation of RAW 264.7 cells and peritoneal macrophages for 30 min with diterpenes resulted in a dose-dependent reduction of the phagocytic activity towards zymosan particles. When the assays were performed with macrophages activated with LPS/IFN-γ, an approximately twofold increase of phagocytosis was observed, a response that was attenuated again by the diterpenes, more efficiently in the case of peritoneal macrophages (Figure 4).

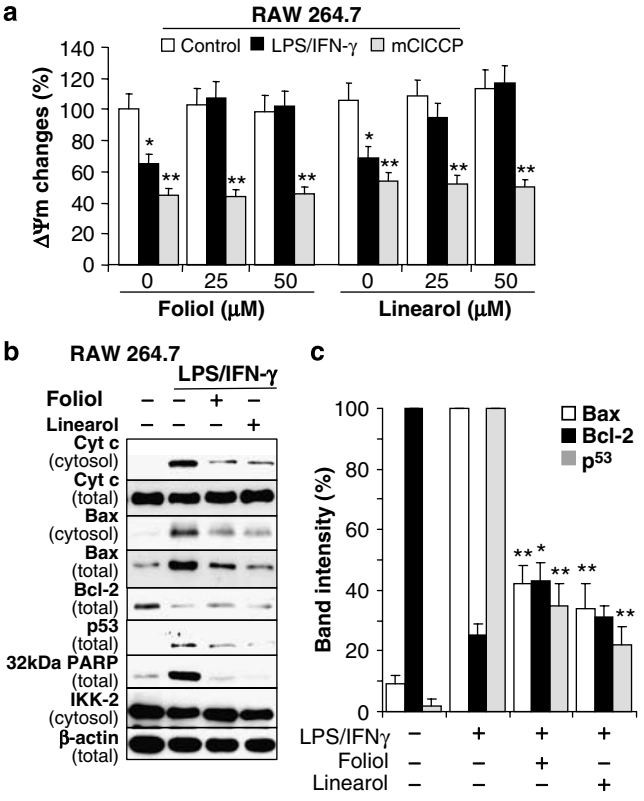

Figure 3 Determination of the mitochondrial inner membrane potential ($\Delta\Psi$m) and levels of apoptosis-related proteins (p53, Bax, Bcl-2, Cyt *c* and PARP) in RAW 264.7 cells treated with LPS/IFN-γ and diterpenes. Cells were preincubated for 30 min with diterpenes (25–50 μM) and then stimulated with LPS/IFN-γ for 8 h. $\Delta\Psi$m was determined by measuring the fluorescence of cells labelled with TMRM; mClCCP (10 μM) was used as positive control (**a**). The levels of cytochrome *c* and Bax were determined by western blot in cytosolic and total cell extracts, and those of p53, Bcl-2 and the cleaved PARP were determined in total extracts. Lane charge was normalized with IKK-2 (cytosol) and β-actin (total). Results show the bands corresponding to a representative experiment (**b**) and the densitometry corresponding to three blots, considering 100% the maximal value for each protein (**c**). Results show the means$\pm$s.d. ($n=4$; **a**) and ($n=3$; **c**). *$P<0.05$, **$P<0.01$ vs the LPS/IFN-γ condition. IFN, interferon; IKK, IκB kinase; LPS, bacterial lipopolysaccharide; M-ClCCP, m-chlorophenylhydrazone carbonyl cyanidine; PARP, poly(ADP-ribose)polymerase; TMRM, tetramethylrhodamine methyl ester.

Discussion

Many terpenes, including diterpenes, triterpenes and sesquiterpenes have proved to possess anti-inflammatory and anticarcinogenic activities (Navarro *et al*., 1997; de las Heras *et al*., 2001; Hwang *et al*., 2001; Lee *et al*., 2002; Nagashima *et al*., 2003; Guzman *et al*., 2005; da Araujo *et al*., 2006) and most of them inhibited NF-κB activity, although the precise mechanism of action has not been fully characterized. In the case of kaurane diterpenes, we have previously identified relevant targets for this inhibition (Castrillo *et al*., 2001). In this context, foliol and linearol inhibited NF-κB activation through a mechanism that involved an impairment of IκB kinase (IKK) activity when assayed in LPS-treated J774

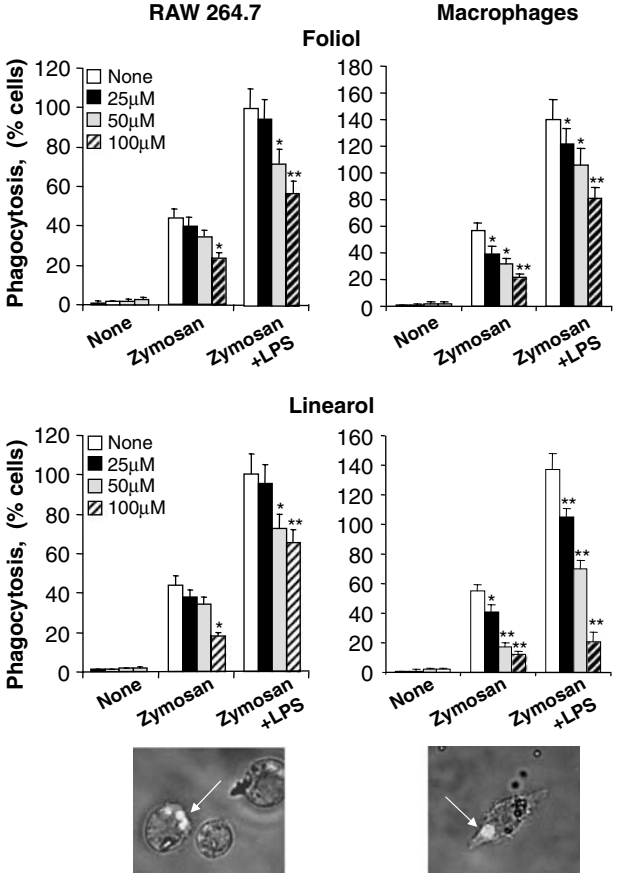

Figure 4 Diterpenes inhibit macrophage phagocytosis. The fluorescence of phagocytosed zymosan particles was determined in RAW 264.7 cells and in macrophages (10^5 cells ml^{-1}) treated with LPS/IFN-γ, diterpenes or combinations of these. Data are the means$\pm$s.d. of four experiments. *$P<0.05$, **$P<0.01$ vs the respective control. A representative picture of macrophages undergoing phagocytosis is shown; arrows indicate the phagocytosed particle. IFN, interferon; LPS, bacterial lipopolysaccharide.

macrophages (Castrillo *et al*., 2001). In addition, these two diterpenes were also inhibitors of production of inflammatory mediators, as they prevented the expression of NOS-2 and COX-2 enzymes. In the present study, we extended the analysis of the biological activities of kaurane diterpenes by demonstrating that foliol and linearol prevented apoptosis and phagocytosis in activated peritoneal macrophages and in the macrophage cell line RAW 264.7.

To summarize the results obtained, apoptosis in macrophages after LPS/IFN-γ activation is mainly dependent of NO synthesis and in fact, when NOS-2 activity was inhibited, the percentage of apoptotic cells diminished dramatically (Hortelano *et al*., 1999). The doses of foliol and linearol used in this work exerted a significant inhibition of NO synthesis in RAW264.7 cells and in peritoneal macrophages, resulting in an impairment of apoptosis and phagocytosis. With respect to the mechanisms involved in the protection from apoptosis, these diterpenes interfered at different levels, including an attenuated rise of pro-apoptotic p53 and Bax levels at the time that the release of cytochrome *c* from the mitochondria to the cytosol and the fall in $\Delta\Psi$m were markedly prevented. In agreement with these data, the activation of caspase-3 measured in intact cells using specific substrates, as well as the cleavage of PARP, a physiological substrate, were significantly inhibited in activated macrophages treated with the diterpenes. In this regard, the activation of caspase-3 in LPS/IFN-γ-treated cells was inhibited by kaurene diterpenes. Biochemical analysis of the activation of caspase-9 and -8 showed that apoptosis was induced via activation of the mitochondrial pathway (caspase-9), without effects on caspase-8 activation (data not shown). Moreover, the observation that overnight treatment with diterpenes, in the absence of LPS/IFN-γ stimulation, resulted in a more potent inhibition of apoptosis indicates that, in addition to the effects on early pro-inflammatory signalling, these molecules confer additional protective mechanisms to macrophages which nature remains to be established.

In addition to the anti-apoptotic effects, our data show that foliol and linearol decrease the phagocytic activity of both resting and activated macrophages, an effect whose pathophysiological consequences remain to be established.

Certain previously studied kaurane diterpenes, all possessing an enone group, have been reported to have pro-apoptotic activity through activation of caspase-8 and -3 (Nagashima *et al*., 2003; Kondoh *et al*., 2004; Suzuki *et al*., 2004a, b). By contrast, foliol and linearol lacking this chemical group have shown protective effects on apoptosis.

In conclusion, our results show that the kaurane diterpenes, foliol and linearol, interact with the mitochondrial pathway protecting macrophages from NO-dependent apoptosis. In addition, these diterpenes can reduce phagocytosis in cultured peritoneal macrophages and in macrophage cell lines.

Acknowledgements

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Conflicts of interest

The author state no conflict of interest.

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