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Inhibition of NOS-2 expression in macrophages through the inactivation of NF-κB by andalusol

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1 Andalusol, *ent*-6α,8α,18-trihydroxy-13(16),14-labdadiene, is a naturally occurring diterpene, isolated from *Sideritis foetens* (Lamiaceae). This compound exhibited therapeutic activity when evaluated in *in vivo* models of paw and ear inflammation (Navarro *et al*., 1997: *Z. Naturforsch*., **52**, 844-849). The pharmacological effects of this diterpene have been analysed on the activation of the macrophage cell line J774 with lipopolysaccharide (LPS) and interferon-γ (IFN-γ).

2 Incubation of J774 macrophages with andalusol (0.1–100 μM) inhibited the synthesis of nitrite caused by LPS (1 μg ml^{-1}) in concentration and time-dependent manners. The maximal inhibition was observed when andalusol was added 30 min before LPS stimulation and decreased progressively as the interval between andalusol and LPS challenge increased up to 14 h.

3 Incubation of J774 cells with LPS resulted in the expression of NOS-2 protein (130 kDa) as identified by Western blot analysis. The levels of this enzyme decreased significantly in the presence of andalusol (IC_{50} = 10.5 μM), suggesting that this diterpene inhibited NOS-2 expression.

4 Andalusol inhibited nuclear factor κB activation, a transcription factor necessary for NOS-2 expression in response to LPS and IFN-γ. This compound also inhibited the degradation of IκBα favouring the retention of the inactive NF-κB complexes in the cytosol.

5 Related compounds to andalusol but lacking the polyol groups were less effective inhibiting NOS-2 expression in LPS-activated macrophages. The present findings provide a mechanism by which the anti-inflammatory properties of this diterpene could be mediated.

Keywords: Andalusol; diterpene; nitric oxide; inducible nitric oxide synthase; transcription factor NF- κB

Abbreviations: Andalusol, *ent*-6α,8α,18-trihydroxy-13(16),14-labdadiene; IL, interleukin; LPS, *E. coli* lipopolysaccharide; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; NOS-2, inducible nitric oxide synthase; PBS, phosphate buffered saline; 1400W, N-(3-(aminomethyl)benzyl)acetamidine

Introduction

Studies on natural products obtained from plants of the genus *Sideritis* (Lamiaceae), mainly flavonoids and terpenes, have shown that they possess important anti-inflammatory activity as assessed using several *in vivo* models of inflammation (Barberan *et al*., 1987; Navarro *et al*., 1997; Villar *et al*., 1984).

Andalusol, a labdane diterpene isolated from an anti-inflammatory extract of *Sideritis foetens* exerted *in vivo* anti-inflammatory activity tested in different inflammation models in mice (carrageenan-induced paw oedema and TPA-induced ear oedema). Oral administration of andalusol inhibited oedema formation, specially late-phase of paw inflammation (5–7 h after carrageenan injection). Andalusol also exerted topical anti-inflammatory activity 4 h after TPA ear application, inhibiting oedema formation and cell infiltration. This compound affected various leukocyte functions and decreased histamine release from mast cells (Navarro *et al*., 1997).

Activation of macrophages with pro-inflammatory cytokines and bacterial cell wall components promotes the synthesis and release of large amounts of nitric oxide (NO), eicosanoids and bioactive lipids such as prostaglandins and leukotrienes, mediators involved in the inflammatory onset (Dugas *et al*., 1995; Albina & Reichner, 1998; DeWitt, 1991; MacMicking *et al*., 1997; Moncada *et al*., 1991). Recently, inhibitors of inducible nitric oxide synthase (NOS-2), the isoenzyme responsible for the high-output NO synthesis, have been proposed as anti-inflammatory agents, mainly because inhibition of exacerbated NO formation may be of therapeutic benefit in these disorders (Xie & Nathan, 1994; Shiloh *et al*., 1999; MacMicking *et al*., 1997; Hierholzer *et al*., 1998). Moreover, a role for NOS-2 has been reported in *in vivo* inflammation models, such as carrageenan-induced hindpaw inflammation in the rat (Handy & Moore, 1998).

Pharmacological, genetic and biochemical analysis of the pathways involved in the expression of NOS-2 showed that activation of NF-κB is an essential, but not sufficient requirement for the expression of this enzyme in mice (Xie & Nathan, 1994; Spink *et al*., 1995; Lowenstein *et al*., 1993; Lyons *et al*., 1992; MacMicking *et al*., 1997; Diaz-Guerra *et al*., 1996). Moreover, transient activation of NF-κB constitutes an important step in the course of several immune and inflammatory responses and plays a key role in the regulated expression of several proinflammatory mediators including cytokines such as IL-1β, IL-6, and IL-8 (Baeuerle & Baichwal, 1997; Baeuerle, 1998; Ghosh *et al*., 1998), and cell adhesion molecules (Lentsch *et al*., 1999; Boyle *et al*., 1998). Because of this pivotal role of NF-κB activation in a variety of inflammatory conditions, this transcription factor is a relevant target for the pharmacological action of anti-inflammatory molecules (Baeuerle & Baichwal, 1997). One potential source for such inhibitors is

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the area of natural products. In this regard, we have studied the effect of andalusol on some macrophage functions in an attempt to understand its mechanism of action as an anti-inflammatory molecule. We have also tried to identify the structural features of diterpenes required for inhibition of NOS-2, by comparing the structure of andalusol with other chemically related diterpenes lacking the ability to inhibit efficiently NOS-2 expression in this model. Our results show that andalusol impairs the activation of NF-κB in J774 macrophages challenged with LPS or with combinations of LPS plus IFN-γ, but this molecule did not affect the activity of the enzyme. These data are compatible with the anti-inflammatory properties of andalusol since this molecule acts through the inhibition of genes requiring NF-κB activation to be expressed.

Methods

Chemicals

Andalusol was isolated from the acetone extract of *Sideritis foetens* aerial parts as described before (Navarro *et al*., 1997). Recombinant murine IFN-γ was obtained from Genzyme. [α-^{32}P]-deoxyCTP was obtained from Amersham (Bucks, U.K). Cell culture reagents were from Life Technologies (Barcelona, Spain). Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The rest of the reagents were purchased from Sigma Chemicals Co. (St. Louis, U.S.A.).

J774 cell culture

The murine macrophage cell line J774, obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, U.K.), was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% foetal calf serum.

Determination of NO synthesis

Cells were plated in 96-well culture plates at a density of 0.5×10^6 cells ml^{-1} until they reached confluence. To induce NOS-2, fresh culture medium containing stimulants was added. Nitrite accumulation in the medium was measured after 24 h, except otherwise stated. Andalusol was added 30 min prior to LPS and IFN-γ challenge. NO formation was measured as the stable end product nitrite in culture supernatant with the Griess reagent (Green *et al*., 1982). NO_2^- concentration was calculated by comparison with OD_{540} of standard solutions of $NaNO_2$ prepared in culture medium.

Measurement of cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT to formazan (Pang *et al*., 1996). J774 macrophages in 96-well plates were treated with andalusol and pro-inflammatory stimuli. 24 h later, the medium was aspirated and cells were incubated with MTT (0.2 mg ml^{-1}) for 1 h at 37°C. The culture medium was removed and cells were solubilized in DMSO (100 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD_{550} in a microplate reader. Viability was set as 100% in untreated cells.

Preparation of cytosolic and nuclear extracts

A modified procedure based on the method of Schreiber (1989) was used. The macrophage cell layers were washed twice with ice-cold PBS, scraped off and collected by centrifugation. Cell pellets were homogenized with 100 μl of buffer A (mM): Hepes 10, pH 7.9, EDTA 1, EGTA 1, KCl 10, dithiothreitol 1, phenylmethylsulfonyl fluoride 0.5 (2 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 2 μg ml^{-1} TLCK) NaF 5, $NaVO_4$ 1, Na_2MoO_4 10). After 10 min at 4 °C, Nonidet P-40 was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s and nuclei were sedimented by centrifugation at $8000 \times g$ for 15 min. Aliquots of the supernatant were stored at −80°C (cytosolic extract) and the nuclei pellet was resuspended in 50 μl of buffer A supplemented with 20% glycerol,

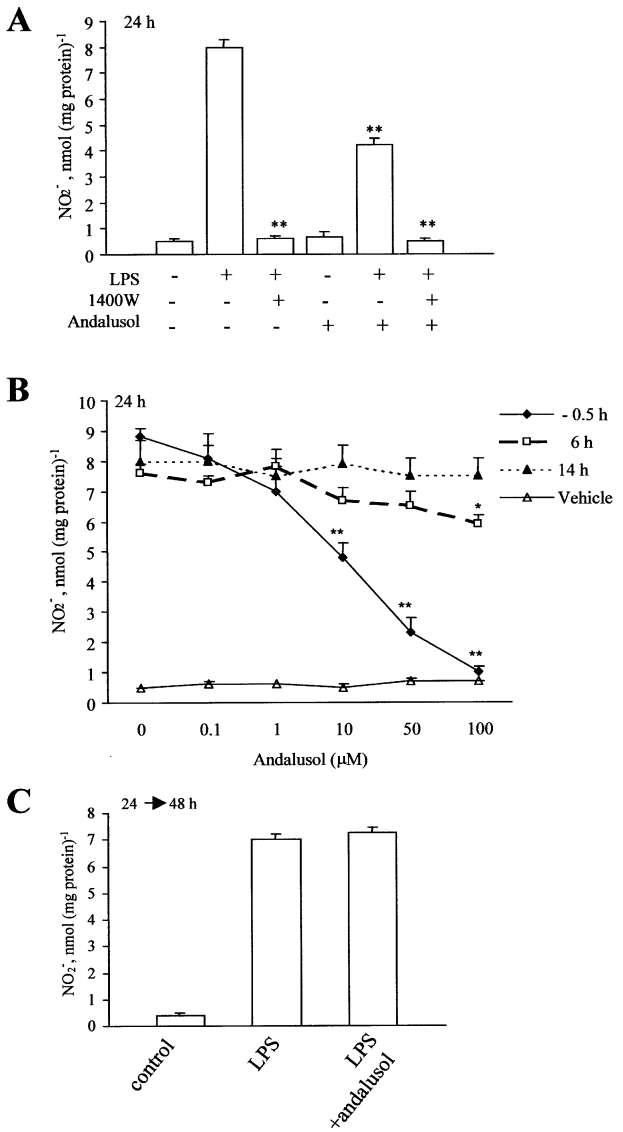


Figure 1 Effect of andalusol on nitric oxide synthesis in J774 cells stimulated with LPS. Macrophages were stimulated with LPS (1 μg ml^{-1}), 1400 W (100 μM), andalusol (10 μM) or combinations of these (A). Andalusol (0.1–100 μM) was added to the cells 0.5 h before or 6 and 14 h after LPS challenge and the concentration of nitrite in the culture medium was determined (B). Cells stimulated for 24 h with LPS were incubated with 50 μM andalusol for another 24 h period and the amount of nitrite accumulated was measured (C). Data are expressed as mean ± s.e.mean of three experiments. *$P<0.05$, **$P<0.01$ with respect to control group (LPS alone at time 0).

0.4 M KCl and gently shaken for 30 min at 4°C. Nuclear proteins were extracted by centrifugation at 13,000 × *g* for 15 min, and aliquots of the supernatant were stored at −80°C. Proteins were measured by the Lowry method (Peterson, 1979). All steps of cell fractionation were conducted at 4°C.

Electrophoretic mobility shift analysis

Synthetic oligonucleotides were prepared using a Pharmacia oligonucleotide synthesizer. The oligonucleotide sequence corresponding to the consensus NF-κB binding site (nucleotides −978 to −952) of the murine NOS-2 promoter was 5′-TGCTAGGGGGATTTTCCCTCTCTCTGT-3′ (Xie *et al*., 1993; Lowenstein *et al*., 1993). Oligonucleotides were annealed with their complementary sequence by incubation for 5 min at 85°C in (mM): Tris-HCl 10, pH 8.0, NaCl 50, $MgCl_2$ 10, dithiothreitol 1. Aliquots of 50 ng of these annealed oligonucleotides were end-labelled with Klenow enzyme in the presence of 1.85 MBq of [α-^{32}P]-deoxyCTP and the other unlabelled dNTPs in a final volume of 50 μl. DNA (1 kBq) was used for each binding assay of nuclear extracts as follows: 5 μg of nuclear proteins were incubated for 15 min at 4°C with the DNA probe and 1 μg of poly(dI-dC), 5% glycerol and (mM) EDTA 1, KCl 100, $MgCl_2$ 5, dithiothreitol 1, Tris-HCl 10, pH 7.8, in a final volume of 20 μl. The DNA-protein complexes were separated on native 6% polyacrylamide gels in 0.5% (v v^{-1}) Tris-borate-EDTA buffer (Velasco *et al*., 1997). Supershift assays were conducted after incubation of the nuclear extract with the Abs (0.5 μg of anti-murine-p50, -p65 and -c-Rel, respectively) for 1 h at 4°C followed by EMSA.

Western blot analysis

Cytosolic and nuclear protein extracts were obtained as described previously for the electrophoretic mobility shift assay (EMSAs). Equal amounts of protein were mixed 1 : 1 with sample buffer (Tris-HCl 60 mM, pH 6.8; 25% glycerol, 2% SDS, β-mercaptoethanol 14 mM, and 0.1% bromophenol blue and boiled for 5 min before electrophoresis was performed. Proteins (30 μg per lane of cytosolic extracts) were size-separated in 7.5% (NOS-2 enzyme) or 10% SDS–PAGE (IκBα, IκBβ). The gels were processed against the murine Ags and after blotting onto a polyvinylidene difluoride membrane, proteins recognized by the antibodies were revealed following the ECL technique (Amersham). Autoradiographs were quantified by laser densitometry (Molecular Dinamics), and several time expositions were analysed to ensure the linearity of the band intensities (Velasco *et al*., 1997).

Statistical evaluation

All values in the figures and text are expressed as mean ± s.e.mean of *n* experiments. Statistical significance was estimated by Student's *t*-test for unpaired observations. A *P* value <0.05 was considered significant.

Results

Andalusol inhibits LPS-induced nitrite production and NOS-2 expression

Activation of J774 macrophages for 24 h with LPS (1 μg ml^{-1}) caused the release of large amounts of NO as deduced by the accumulation of nitrite in the culture medium. This response was due to the expression of NOS-2 enzyme since the synthesis of NO was completely inhibited by the NOS-2 specific inhibitor 1400W (Figure 1A). Andalusol assayed at 10 μM in the absence of LPS, did not alter the basal nitrite concentration, but inhibited 47% of the NO synthesis observed in LPS-activated cells. Pretreatment of macrophages for 30 min with various concentrations of andalusol (0.1 – 100 μM) caused a concentration-dependent reduction of NO generation (IC_{50} = 10.5 μM). The DMSO concentration used as andalusol vehicle had no effect on nitrite production. The highest concentration of andalusol tested (100 μM) nearly abolished NO synthesis (Figure 1B). In J774 macrophage challenged with LPS, nitrite appears at about 8 h and the rate of production remains constant for at least 24 h. Experiments performed by adding andalusol 6 or 14 h after LPS stimulation showed that the inhibitory response decreased at 6 h (22% of inhibition at 100 μM) and was abolished when andalusol was added 14 h after LPS stimulation (Figure 1B). Moreover, in cells activated with LPS for 24 h, addition of 50 μM andalusol and the culture maintained for another 24 h

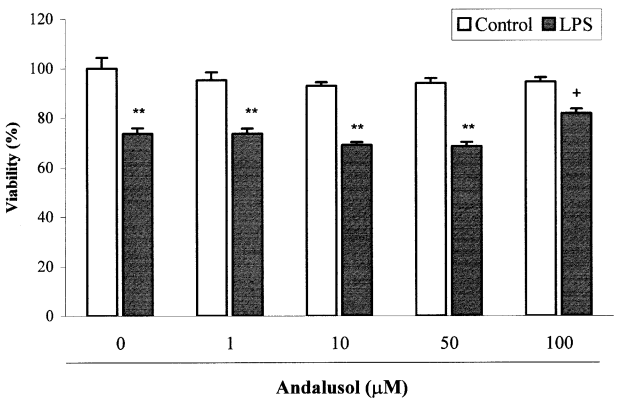


Figure 2 Effect of andalusol on the viability of J774 cells. Andalusol was added 30 min before exposure to LPS (1 μg ml^{-1}) and cell viability was determined by mitochondrial-dependent MTT reduction after 24 h of incubation. $^{**}P<0.01$ with respect to control group, + $P<0.05$ with respect to LPS-treated group.

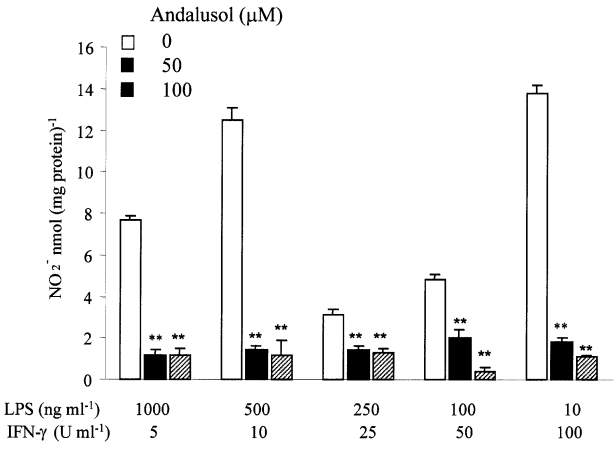


Figure 3 Effect of andalusol on nitrite formation in cells stimulated with LPS and IFN-γ mixtures. Cells were pre-incubated with andalusol 30 min prior to challenge with combinations of LPS and IFN-γ and the accumulation of nitrite was determined after 24 h of culture. Data are expressed as mean ± s.e.mean of four experiments. $^{**}P<0.01$ with respect to the condition in the absence of andalusol.

incubation period was unable to inhibit the nitrite accumulation in the culture medium (Figure 1C). These results indicate that andalusol did not cause direct inhibition of NOS-2 activity, but rather affected the expression of the enzyme during the initial 0–6 h after LPS stimulation.

Based on MTT assays, viability was not reduced significantly after 24 h of incubation with andalusol concentrations up to 100 μM, nor decreased cellular respiration (Figure 2). Therefore, andalusol does not induce cytotoxicity and/or non-specific depression of cellular respiration in J774 macrophages. Moreover, since LPS induces a loss of cell viability within 24 h of culture, it was of interest to evaluate whether andalusol might protect against LPS-induced damage of these cells. As shown in Figure 2, andalusol (100 μM) added 30 min before LPS, exerted a moderate but significant protective effect against LPS-induced cytotoxicity (see Discussion).

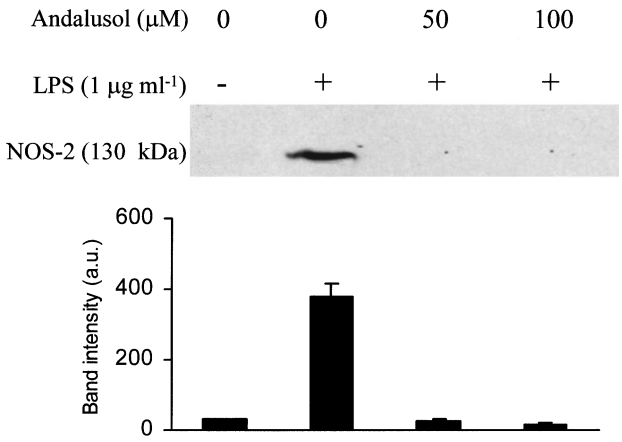


Figure 4 Effect of andalusol on NOS-2 protein levels in activated J774 cells. Macrophages were pre-treated with andalusol (50, 100 μM) followed by LPS (1 μg ml^{-1}) challenge. NOS-2 protein levels were determined by Western blot in cell extracts obtained after 24 h of culture. Results show a representative blot and the mean±s.e.mean of the corresponding band intensities from three experiments.

To further investigate the mechanism responsible for the inhibition of NO synthesis by andalusol, an experiment was performed in which cells were stimulated with different LPS/IFN-γ combinations and the effect of this diterpene was determined. As shown in Figure 3, IFN-γ synergized with LPS causing an important enhancement of nitrite accumulation (12.7 ± 0.6 nmol (mg protein)$^{-1}$, when cells were stimulated with 500 ng ml^{-1} LPS and 10 U ml^{-1} IFN-γ). Andalusol assayed at 100 μM was able to reduce NO synthesis up to control values regardless of the synergistic action of IFN-γ in serial combinations of LPS and IFN-γ (Figure 3).

To confirm that the inhibitory effect of andalusol on NO synthesis was due to an impaired expression of NOS-2, the protein levels of this enzyme were determined by Western blot analysis of cell lysates. J774 cells treated with LPS (1 μg ml^{-1}) expressed NOS-2 but in the presence of 50 and 100 μM of andalusol, the protein levels were undetectable after 24 h of culture (Figure 4).

Effect of andalusol on NF-κB activty

To better characterize the mechanisms involved in the inhibition exerted by andalusol on NOS-2 expression, NF-κB activity was analysed by EMSA using an oligonucleotide containing the κB motif corresponding to the distal κB site present in the murine NOS-2 promoter (Lowenstein *et al*., 1993; Spink *et al*., 1995). Nuclear protein extracts were prepared from cells incubated with different concentrations of andalusol and subsequently stimulated with LPS (1 μg ml^{-1}) for periods of 1 and 2 h. A negligible level of NF-κB binding to DNA was detected in nuclear proteins from untreated macrophages. Conversely, retarded bands were clearly observed following LPS activation. As Figure 5 shows, NF-κB activation exhibited a peak 1 h after LPS challenge. Characterization of the proteins retained in EMSA by supershift analysis revealed the presence of p50–p65 heterodimers and p50 homodimers in the upper and lower bands, respectively. The NF-κB activity was inhibited 80% at 1 h in the presence of 50 μM andalusol. However, this inhibitory effect of andalusol was less marked when extracts were

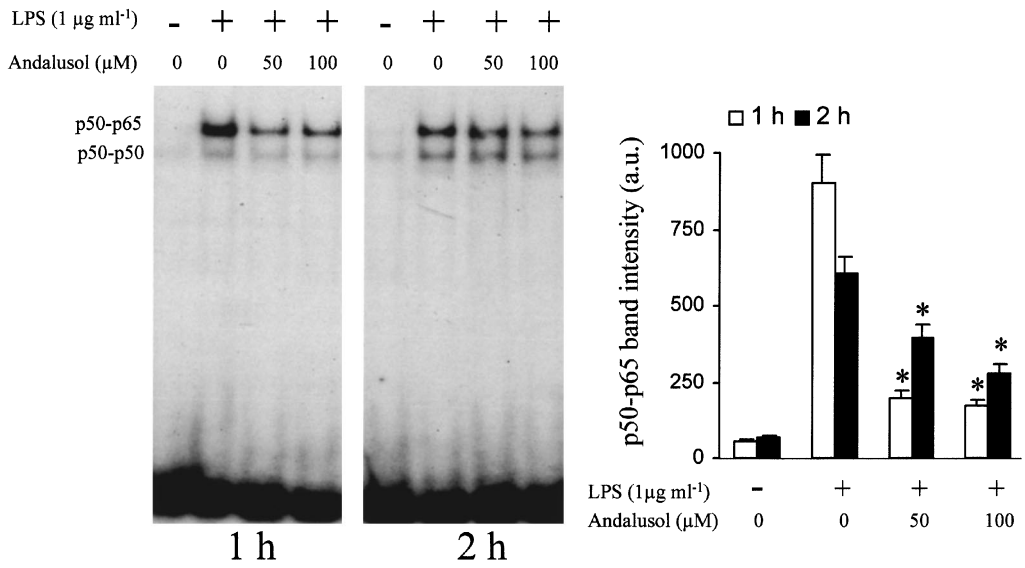


Figure 5 Effect of andalusol on NF-κB activity in LPS-treated J774 cells. NF-κB activity was measured by EMSA using nuclear extracts (5 μg of protein) and following the binding to the κB motif of the NOS-2 promoter. J774 cells were preincubated for 30 min with indicated concentrations of andalusol, followed by stimulation with LPS (1 μg ml^{-1}) for 1 and 2 h. Results show a representative EMSA and the mean±s.e.mean of the intensity corresponding to the upper band. *$P<0.05$ with respect to cells treated with LPS in the absence of andalusol.

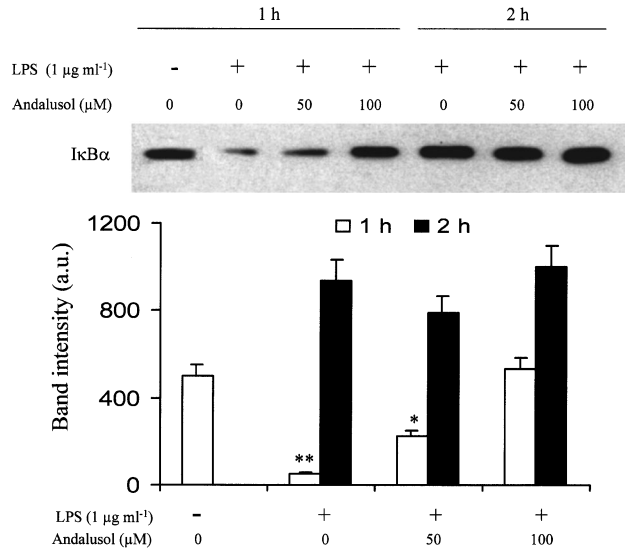
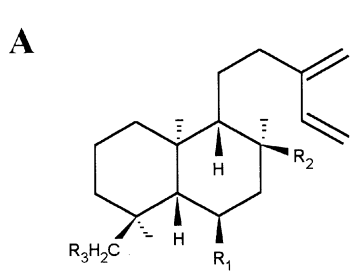


Figure 6 Andalusol inhibits IκBα degradation in LPS-activated J774 cells. The IκBα protein levels were determined by Western blot at the indicated times. Results show a representative blot and the mean ± s.e.mean of the band intensity from three experiments. $*P<0.05$ with respect to the LPS condition in the absence of andalusol.

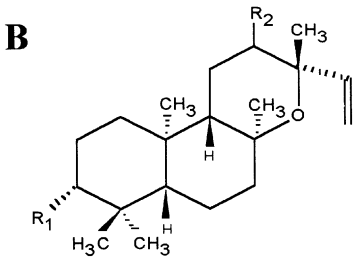
prepared from cells incubated with LPS for 2 h (40% inhibition).

Effect of andalusol on IκB protein levels

NF-κB is retained in the cytosol through the interaction with inhibitory proteins termed IκB (Baeuerle, 1998; Baeuerle & Baichwal, 1997). Since NF-κB activation is dependent on IκB phosphorylation and degradation the measurement of IκB protein levels offers additional information to determine the fate of NF-κB. To analyse the effect of andalusol on IκB levels the amount of IκBα and IκBβ in the cytosol was determined by Western blot. Unstimulated cells had important levels of IκBα, whereas IκBβ was negligible. When cells were activated for 1 h with LPS, a rapid and important degradation (90% of the control value) of IκBα was observed (Figure 6). Treatment of cells with andalusol (50, 100 μM) prevented, concentration-dependently the LPS-induced IκBα degradation suggesting that the inhibitory action of this diterpene is due to the abrogation of IκBα phosphorylation and/or degradation, therefore retaining the inactive NF-κB complexes in the cytosol. When IκBα levels were determined at 2 h, an important up-regulation of this protein was observed, suggesting a rapid resetting of its levels.



	R1	R2	R3
ent-6α,8α,18-trihydroxy-13(16), 14-labdadiene (*a*)	OH	OH	OH
ent-8α,hidroxy-13(16), 14-labdadiene (*b*)	H	OH	H



	R1	R2
ent-3β hydroxy-13-epi-manoyl oxide (*c*)	OH	H
ent-12α–acetoxy-13-epi-manoyl oxide (*d*)	H	OCH_2CH_3

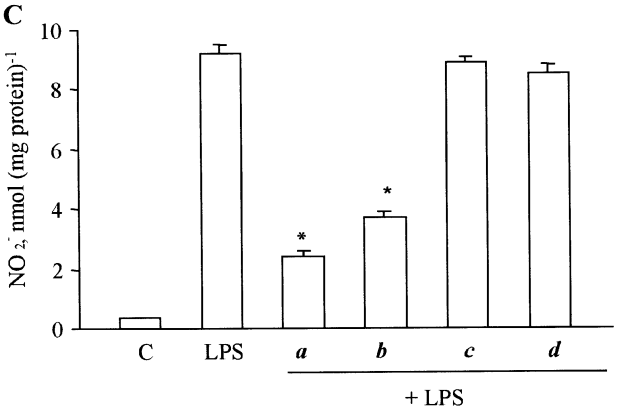


Figure 7 Chemical structures of diterpenes and effect on NO synthesis in LPS-activated J774 cells. (A) Labdane-type: andalusol (*ent*-6α,8α,18-trihydroxy-13(16),14-labdadiene) and *ent*-8α-hydroxy-13(16),14-labdadiene and (B) Manoyl oxide-type: ribenol (*ent*-3β-hydroxy-13-*epi*-manoyl oxide) and *ent*-12α-acetoxy-13-*epi*-manoyl oxide. The effect of these diterpenes (assayed at 50 μM) on NO synthesis in J774 cells challenged with LPS (1 μg ml^{-1}) for 24 h was determined (C). Results show the mean ± s.e.mean of three experiments. $*P<0.05$ with respect to andalusol.

Comparative effect of structurally related diterpenes on abrogation of macrophage NOS-2 expression

To evaluate the ability of closely related diterpenes on the activation of J774 cells challenged with LPS the effect of two series of molecules were assayed (Figure 7A,B). The hydrophilic polyol labdadiene andalusol was significantly more potent (15%) than the hydrophobic counterpart in the inhibition of nitrite release when assayed at 50 μM. However, the related manoyl oxides failed to inhibit NOS-2 expression under these conditions.

Discussion

Several biological actions have been reported for terpenes including antimicrobial, anti-inflammatory and antiviral effects (Navarro *et al*., 1997; Singh *et al*., 1999). The present study focused on the evaluation of the action of andalusol, a labdane-type diterpene, on macrophage expression of NOS-2, and could explain previous work on the anti-inflammatory properties of this molecule carried out in animal models (Navarro *et al*., 1997).

We studied the action of andalusol on the synthesis of NO by activated J774 macrophages, and our results showed a very potent effect for this diterpene inhibiting both LPS and LPS plus IFN-γ-dependent expression of NOS-2. The observation that the maximal effects were obtained when andalusol was added prior to macrophage challenge suggests an inhibitory action of this diterpene on the transcriptional mechanism leading to NOS-2 expression. Interestingly, andalusol at concentrations up to 100 μM was unable to inhibit NOS-2 activity once expressed in the activated cells, or when assayed *in vitro* using the purified enzyme (not shown). Moreover, immunoblots using specific antibodies showed a decrease of NOS-2 protein levels. Similar effects on NOS-2 expression have also been described for another labdane-type diterpene (Pang *et al*., 1996), but this was notably less potent. Moreover, manoyl oxide-type diterpenes that are very close chemically to the labdane skeleton, but differing in the cyclic structure of the epoxide ring were unable to inhibit NO synthesis in these cells. These results suggest the relevance of the -OH group in R2 (C_8 position of the labdane) for the inhibition of NOS-2 expression. It is to note the observation that the presence of an epoxide ring in sesquiterpenes appears to potentiate their ability to inhibit NF-κB, whereas in diterpenes results in a loss of biological activity. However, it remains to be clarified whether this structural feature may influence the potency among labdane diterpenes (Singh *et al*., 1999). In this regard, natural diterpenes exhibit different chemical structures around the diterpene skeleton that are critical for their biological properties; for example, whereas taxol activates NF-κB and NOS-2 expression (Kirikae *et al*., 1996; Hwang & Ding, 1995), others such as forskolin decreased LPS-induced NF-κB activity and NOS-2 expression in Kupffer cells (Mustafa & Olson, 1998), through an increase of cyclic AMP levels (Ollivier *et al*., 1996).

Previous work reported that the anti-inflammatory effects of some terpenes such as sesquiterpene lactones can be assigned, at least to a certain extent, to the inhibition of the activity of some transcription factors (Hehner *et al*., 1998; Rungeler *et al*., 1998). However, these effects have not yet been reported for diterpenes, and it is notable that the IC_{50} value for andalusol (10 μM) is similar to that observed for sesquiterpene lactones (Hehner *et al*., 1998). Triterpenes have been studied in depth and important inhibition of NOS-2 and cyclooxygenase-2 expression have also been observed (Suh *et al*., 1998, 1999).

Our data show an important effect of andalusol on the inhibition of NF-κB activation. Since this transcription factor participates in the regulation of the expression of multiple immediate early genes involved in the immune, acute phase and inflammatory responses, the action of andalusol on this target might explain part of the anti-inflammatory actions of this molecule (Ghosh *et al*., 1998; Baeuerle & Baichwal, 1997). In light of the role of NF-κB as a co-ordinating regulator in the expression of a variety of rapid-response genes as NOS-2 gene, therapeutic regulation of this factor might likely be of benefit in the treatment of immune and inflammatory disorders.

NF-κB activation has a key role in the regulation of NOS-2 expression as deduced by genetic and biochemical analysis, at least when the expression of the enzyme depends on the action of pro-inflammatory cytokines, bacterial cell wall products and type II IFN (Diaz-Guerra *et al*., 1996; Lowenstein *et al*., 1993; Xie *et al*., 1993; Moncada *et al*., 1991; Dugas *et al*., 1995). Multiple signalling pathways have been described in the process of NF-κB activation in response to LPS, including protein tyrosine kinases (Novogrodsky *et al*., 1994; DeFranco *et al*., 1995), and an enhancement of the synthesis of reactive oxygen species (Imbert *et al*., 1996), which makes difficult the exact identification of targets inhibited by andalusol. One key step in the activation process is the phosphorylation, ubiquitinylation and subsequent degradation of the inhibitory molecules IκB, allowing migration of NF-κB to the nucleus. Evidence of the ability of andalusol to inhibit NF-κB activity was seen in EMSA, since nuclear extracts from LPS-stimulated J774 cells pre-treated with andalusol showed a reduced binding of NF-κB to an oligonucleotide containing a κB site.

NF-κB activity depends ultimately on the balance between the rates of degradation and resynthesis of IκB proteins (Baeuerle, 1998). The results obtained with EMSA analysis agree with those observed by Western blotting of the IκB proteins. Andalusol prevented the degradation of IκBα from the cytosolic fraction inhibiting the transcription of genes dependent on NF-κB activity. This degradation was very rapid since a complete loss of immunodetected protein in the Western blot was observed at 30 min after stimulation, and is compatible with the reduced time expand in which andalusol is active.

Regarding the mechanism of action of NF-κB inhibitors, two possibilities can be considered: One group of inhibitors exerts its effect by scavenging reactive oxygen species, and this appears to be the mechanism of action of antioxidant molecules (Schreck *et al*., 1991; Baeuerle & Baichwal, 1997; Mihm *et al*., 1991; Lee *et al*., 1997; Kopp & Ghosh, 1994). Another group interferes with the phosphorylation and degradation of IκB-family members by affecting the signalling involved in specific phosphorylation or the activity of the 26 S proteasome (Traenckner *et al*., 1994; Cobb *et al*., 1996; DiDonato *et al*., 1997) or favouring the accumulation of IκB as observed for the long-term action of glucocorticoids (Auphan *et al*., 1995). This diterpene has no antioxidant properties as it does not interfere with the generation of free radicals (Navarro *et al*., 1997), and it is unlikely an action through the glucocorticoid receptors, since this has been investigated in detail for triterpenes (Suh *et al*., 1998, 1999). Therefore, andalusol might inhibit a step leading to the activation of the IκB kinase (IKK), or possibly the IKK itself, but further work is required to address this question (Song *et al*., 1997; DiDonato *et al*., 1997). Indeed, activation of monocytes with LPS stimulates specifically IKK2, and this kinase exhibits a transient activation kinetics compatible with

the restricted pattern of activation of NF-κB in these cells (Delhase *et al*., 1999; O'Connell *et al*., 1998).

Finally, in this work we studied the action of andalusol on NF-κB activity, but the experiments carried out after simultaneous stimulation with LPS and IFN-γ, strongly suggest that in addition to NF-κB, it is likely the possibility of an inhibitory action on the IFN-γ signalling. Indeed, this has been described for triterpenes (Suh *et al*., 1998) and if this would be the case of andalusol, it opens additional perspectives for the study of the therapeutic action of these molecules.

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