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New insights into the mechanism of action of the anti-inflammatory triterpene lupeol

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Abstract

The pentacyclic triterpene lupeol has been studied for its inhibitory effects on murine models of inflammation and peritoneal macrophage functions in-vitro. Lupeol (0.5 and 1 mg/ear) administered topically suppressed the mouse ear oedema induced by 12-*O*-tetradecanoylphorbol acetate (TPA), being less effective on ear oedema induced by arachidonic acid. Quantitation of the neutrophil specific marker myeloperoxidase demonstrated that its topical activity was associated with reduction in cell infiltration into inflamed tissues. When tested in-vitro, lupeol significantly reduced prostaglandin E_2 (PGE_2) production from A23187-stimulated macrophages, but failed to affect leukotriene C_4 release. It was a weak inhibitor of nitrite release, but dose-dependently suppressed PGE_2. Cytokine production (tumour necrosis factor-α and interleukin-1β) was inhibited in the range 10–100 μM in lipopolysaccharide-treated macrophages. This study demonstrated that lupeol possessed anti-inflammatory activity which was likely to depend on its ability to prevent the production of some pro-inflammatory mediators.

Introduction

Pentacyclic triterpenes have a wide distribution in plants and represent a large group of compounds with highly-interesting biological actions, including anti-inflammatory properties (Konoshima et al 1992; Kapil & Sharma 1995; Safayhi & Sailer 1997; Cantrell et al 1999). This type of compound has been studied in depth and an important inhibition of the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) enzymes, protein kinase (PKA) and serine proteases have been observed with some triterpenes (Suh et al 1998; Hasmada et al 1999; Honda et al 1999; Rajic et al 2000). Lupeol is a naturally occurring lupane triterpene found in various plants. Lupeol has been shown to reduce the inflammatory response of adjuvant arthritis in rats and has been demonstrated to have immunomodulating properties (Geetha et al 1998; Geetha & Varalakshmi 1999a, b), but no pharmacological data on its mechanism of action have been reported.

Inflammation is a complex pathophysiological process. It is mediated by a variety of signalling molecules produced by leucocytes, macrophages, mast cells, platelets, and lymphocytes, as well as by the activation of complement factors which bring about oedema formation as a result of extravasation of fluid and proteins and accumulation of leucocytes at the inflammatory site. Macrophages play a

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crucial role in modulating the initiation and perpetuation of the inflammatory response. Activation of these cells causes the release of eicosanoids, nitric oxide (NO) and pro-inflammatory cytokines, including tumour necrosis factor (TNF-α) and interleukin (IL-1β) (Moilanen & Vapaatalo 1995; Vane et al 1998). The oxidative metabolism of arachidonic acid leads to the synthesis of prostaglandins and leukotrienes. Cyclooxygenase catalyses the first rate limiting step in the synthesis of prostaglandins and thromboxanes from arachidonic acid. Two cyclooxygenase isoenzymes have been identified. Firstly, COX-1, which is constitutively expressed in a wide variety of tissues and is responsible for the low prostaglandin synthesis required for cell homeostasis. Secondly, COX-2, which is a highly-inducible enzyme that is expressed in the course of inflammation or other cellular stresses and accounts for the important synthesis of prostanoids that occurs in several physiopathological situations such as septic shock and local inflammation of target tissues (Crofford 1997).

NO, which is formed from L-arginine by the NOS enzyme, seems to be involved in both acute and chronic inflammation (McMicking et al 1997). Since the induction of COX-2 and NOS results in the increased synthesis of prostaglandins and NO, selective modulation of these mediators' overproduction might represent a therapeutic goal in different inflammatory pathologies.

Cytokines such as IL-1β and TNF-α are important pro-inflammatory mediators. Small amounts of these cytokines play a protective role acting as a host defence factor in immunologic and inflammatory responses. However, the uncontrolled release of cytokines is the basis for the development of inflammatory diseases including asthma, rheumatoid arthritis, and inflammatory bowel disease (Sekut & Connolly 1996).

In this study, we have investigated the effects of lupeol on some inflammatory responses. Lupeol was tested for acute anti-inflammatory effects in-vivo as well as for its ability to influence the generation of some mediators and cytokines involved in the inflammatory process.

Materials and Methods

Materials

Lupeol was isolated from an extract of *Pimenta racemosa* (Myrtaceae) as described by Fernandez et al (2001). Cell culture reagents were purchased from Life Technologies (Barcelona, Spain). Enzyme-linked immunosorbent assay (ELISA) kits for the determination of prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4) were provided by Cayman Chemical Co (USA). TNF-α and IL-1 β ELISA kits were from Amersham Iberica (Madrid, Spain). The other reagents were purchased from Sigma Chemical Co. (St Louis, MO).

12-*O*-tetradecanoylphorbol acetate (TPA)-induced mouse ear oedema

The protocols were approved by the institutional Animal Care and Use Committee. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals.

Oedema was induced by topical application of 2.5 μg TPA in 20 μL acetone to the right ear of male Swiss mice (25 $\pm$ 5 g). The left ear (control) received acetone only. Lupeol was applied topically in acetone (0.5 or 1 mg/ear) before TPA administration. A reference group was treated with indometacin (0.5 or 1 mg/ear). After 4 h, animals were killed by cervical dislocation and a 6-mm biopsy was obtained from both ears and weighed. The weight increase of the right ear punch over that of the left indicated the oedema (Carlson et al 1985). Ear sections were homogenized in 750 μL saline and after centrifugation at 10 000 *g* for 15 min at 4°C, myeloperoxidase (MPO) activity was measured in supernatants as described by De Young et al (1989).

Arachidonic acid-induced mouse ear oedema

Arachidonic acid (2.0 mg) dissolved in 20 μL acetone was applied to the right ear of mice. Lupeol was applied topically in acetone 30 min before arachidonic acid. The left ear (control) received vehicle only. The oedema was measured 1 h after induction (Carlson et al 1989).

Production of PGE_2 and LTC_4 in calcium ionophore A23187-stimulated macrophages

Macrophages from Swiss male mice were collected after peritoneal lavage with phosphate-buffered saline (PBS) (De las Heras & Hoult 1994). Briefly, cells were suspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and seeded into 24-well plates at a concentration of 0.5×10^6 cells mL^{-1}. After adhering to plates (2 h at 37°C in an atmosphere of 5% CO_2/95% air), non-adherent cells were washed off and then cultured in DMEM without FCS. Cells were pretreated for 30 min

at 37°C with test compounds or vehicle and then stimulated for a further 2 h by adding calcium ionophore A23187 (final concentration 1 μM). The medium was withdrawn from each well and levels of PGE_2 and LTC_4 quantified by using ELISA kits.

Production of nitrite, PGE_2, TNF-α and IL-1β in lipopolysaccharide-activated macrophages

Swiss male mice were injected intraperitoneally with 1 mL thioglycolate broth four days before use (Lopez-Collazo et al 1998). Peritoneal macrophages were prepared as follows. Light ether-anaesthetized mice (4–6 animals) were killed by cervical dislocation and injected intraperitoneally with 5 mL sterile RPMI 1640 medium. The peritoneal fluid was carefully aspirated to avoid haemorrhage and kept at 4°C to prevent the adhesion of macrophages to the plastic. After centrifugation at 200 *g* for 10 min at 4°C, the cell pellet was washed twice with 45 mL ice-cold PBS. Cells were seeded at 1×10^6 mL^{-1} in RPMI 1640 supplemented with 10% FCS. After incubation for 2 h at 37°C in 5% CO_2, non-adherent cells were removed by extensive washing with PBS. Cells were incubated in the same medium (1% FCS) but containing *Escherichia coli* lipopolysaccharide (0.5 μg mL^{-1}) with or without test compounds at 37°C for 24 h. Culture supernatants were used for measurement of NO, PGE_2, TNF-α and IL-1. NO released was assessed spectrophotometrically as the stable end product nitrite in the culture supernatant with the Griess reagent (Green et al 1982). PGE_2, TNF-α and IL-1β were measured by ELISA kits according to the manufacturer's instructions.

Cytotoxicity assays

Lactate dehydrogenase was determined by measuring the rate of oxidation of nicotinamide adenine dinucleotide (NADH) (Bergmeyer & Bernt 1974). Cells treated with Triton X-100 (0.5%) were used for measurement of the total cellular content of lactate dehydrogenase.

Macrophages (5×10^5 cells) in 96-well plates were incubated overnight at 37°C and 5% CO_2/95% air. Cells were exposed to various concentrations of lupeol (0–100 μM) for 20 h under the same incubation conditions. After this, 3-(4-5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, 0.2 mg mL^{-1}) was added and further incubated for 60 min. The medium was removed by aspiration and the cells were solubilized with dimethylsulfoxide (DMSO, 100 μL). The absorbance at 550 nm was read in a microtitre plate reader (Pang et al 1996).

Data analysis

The results are presented as mean ± s.e.m. The level of statistical significance was estimated by one-way analysis of variance followed by the Student's *t*-test.

Results

Mouse ear oedema

Evaluation of the acute anti-inflammatory activity of lupeol was performed in the mouse ear oedema model induced by TPA or arachidonic acid. Topical application of lupeol (0.5 or 1 mg/ear) significantly attenuated the TPA-induced oedema (36.2% and 52.0% of

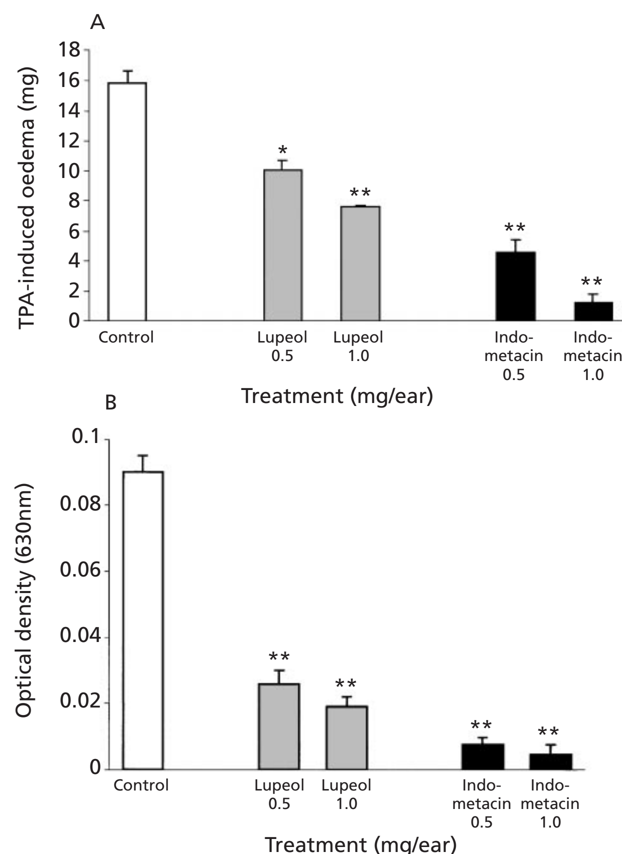

Figure 1 Effect of lupeol and indometacin on (A) TPA-induced mouse ear oedema and (B) myeloperoxidase activity in supernatants of homogenates from TPA-treated ears. Lupeol and indometacin were administered topically at the time of TPA application (2.5 μg/ear). Values are expressed as mean ± s.e.m. from eight mice. *$P < 0.05$, ** $P < 0.01$ vs control.

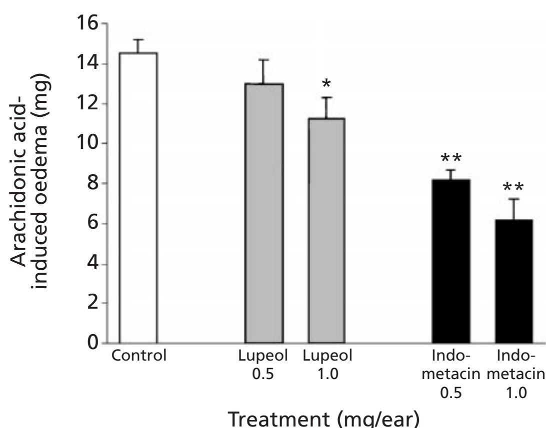

Figure 2 Effect of lupeol and indometacin on arachidonic acid-induced mouse ear oedema. Oedema was expressed as mg weight of ear sections (mean ± s.e.m.) from eight animals. Lupeol and indometacin were administered topically 30 min before arachidonic acid application. $*P < 0.05$, $**P < 0.01$ vs control.

Table 1 Effects of lupeol and reference drugs on PGE_2 and LTC_4 release in calcium ionophore A23187-stimulated peritoneal macrophages.

Treatment	PGE_2 (ng mL^{-1})	LTC_4 (ng mL^{-1})
Cells alone	0.4 ± 0.1	0.2 ± 0.1
A23187	4.1 ± 0.8	3.5 ± 0.3
Lupeol 0.1 μM	3.0 ± 0.5	3.2 ± 0.2
Lupeol 1.0 μM	2.7 ± 0.3	2.9 ± 0.1
Lupeol 10 μM	2.4 ± 0.2*	2.9 ± 0.1
Lupeol 100 μM	2.0 ± 0.3**	2.1 ± 0.1*
Indometacin 10 μM	0.4 ± 0.3**	N.D.
Nordihidroguayaretic acid 10 μM	N.D.	1.2 ± 0.1**

Data are mean ± s.e.m., n = 5–8. $**P < 0.05$, $**P < 0.01$ compared with A23187 control group. N.D., not determined.

inhibition, respectively) (Figure 1A). The observed topical anti-inflammatory activity was confirmed by quantifying the levels of the neutrophil-specific marker MPO, which was extracted from the ear biopsy. A high level of MPO was noted in TPA-treated ears 4 h after induction of inflammation. Figure 1 demonstrates a high correlation between lupeol inhibition of oedema and the neutrophil influx as assessed biochemically by accumulation of MPO. Lupeol significantly decreased MPO activity by 71% and 78.5% at 0.5 and 1 mg/ear, respectively, compared with control animals (Figure 1B). Indometacin was more effective on MPO than on oedema, which was also attenuated.

Inhibition of the 5-lipoxygenase (5-LOX) pathway after topical administration of lupeol to mice was evaluated using the arachidonic acid-induced inflammation model, which is relatively selective for 5-LOX inhibitors. As shown in Figure 2, lupeol and indometacin had less pronounced anti-inflammatory activity in this model.

Production of PGE_2 and LTC_4 release in A23187-stimulated macrophages

Lupeol did not significantly affect mitochondrial reduction of MTT or lactate dehydrogenase release at the concentrations assayed. On MTT assay, it did not show any cytotoxic effect (% viability 97.2 ± 1.5, 94.2 ± 1.3, 92.5 ± 1.7 at 1, 10 and 100 μM, respectively vs 100% viability of untreated cells). Addition of calcium ionophore A23187 to mouse peritoneal macrophages caused the generation of nanogram amounts of eicosanoids via both COX-1 and 5-LOX pathways, measured in terms of immuno-assayable PGE_2 and LTC_4, respectively. Validation of this system for the identification of inhibitors of the two divergent pathways of arachidonate metabolism was obtained by using indometacin, a well-characterized cyclooxygenase inhibitor and nordihidroguayaretic acid, a known inhibitor of 5-LOX that potently reduced LTC_4 synthesis. As shown in Table 1, pretreatment of cells with lupeol (0.1–100 μM) significantly reduced PGE_2 levels, achieving an inhibition percentage of 51.2% at the highest concentration tested (100 μM).

The production of LTC_4 by unstimulated macrophages was < 0.2 ng mL^{-1}. Incubation of these cells with A23187 caused a substantial increase in LTC_4 production. Lupeol did not show any inhibitory effect on LTC_4 release.

Production of nitrite, PGE_2, TNFα and IL-1β in lipopolysaccharide-stimulated macrophages

Incubation of macrophages with lipopolysaccharide (0.5 μg mL^{-1}) greatly increased the production of NO, PGE_2 and cytokines (Table 2).

Lupeol was a weak inhibitor of NO release, as only at the highest dose was nitrite accumulation significantly reduced in the medium. When macrophages were stimulated with the same amount of lipopolysaccharide in the presence of lupeol (1–100 μM) a concentration-dependent inhibition of PGE_2 production was observed (IC50, concentration giving 50% inhibition, 24.3 μM). Cytokine production (TNFα, IL-1β) was significantly reduced by lupeol in the range 10–100 μM.

Dexamethasone, used as reference compound, potently inhibited the four parameters assayed.

Table 2 Effects of lupeol and dexamethasone (reference drug) on nitrite, PGE_2, TNF-α and IL-1β production in lipopolysaccharide-stimulated peritoneal macrophages.

Treatment	Nitrite (μM)	PGE_2 (ng mL^{-1})	TNF-α (ng mL^{-1})	IL-1β (pg mL^{-1})
Cells alone	2.6±0.5	0.7±0.1	3.4±0.3	40.0±1.0
Lipopolysaccharide	43.6±2.6	3.7±0.2	16.1±1.4	200.0±2.0
Lupeol 0.1 μM	53.1±3.6	2.9±0.1	18.8±1.7	210.0±3.0
Lupeol 1.0 μM	49.4±2.7	2.6±0.2*	16.2±1.4	170.0±3.0
Lupeol 10 μM	39.7±2.1	2.5±0.2*	10.7±0.9*	150.0±2.1*
Lupeol 100 μM	17.4±1.2**	1.3±0.1**	5.3±0.9**	90.0±2.0**
Dexamethasone (1 μM)	19.0±3.1**	0.8±0.1**	2.0±0.4**	30.0±1.5**

Data are the mean±s.e.m. (n = 5–8). $*P < 0.05$, $**P < 0.01$ compared with lipopolysaccharide control group. Lupeol was added to cells 30 min before lipopolysaccharide and incubation continued for 24 h.

Discussion

We have investigated the acute anti-inflammatory activity of lupeol and its ability to reduce the production of some inflammatory mediators in-vitro. Mouse ear oedema was used as a model of acute inflammation. Lupeol was able to inhibit experimental acute inflammation after topical application in the TPA-induced ear oedema. It has been well established that the inflammation induced by this agent is related to the activation of protein kinase C (Jacobson et al 1995). Protein kinase C is a family of specific protein kinases, which play a role in a range of signal transduction processes. Protein kinase C activation stimulates cyclooxygenase activity and augments Ca^{2+}-dependent lipoxygenase products, which are involved in vascular permeability in this model. MPO, a haemoprotein located in the azurophil granules of neutrophils has been used as a biochemical marker for neutrophil infiltration into tissues. Lupeol significantly reduced MPO levels in ear homogenates, indicating that a control on leucocyte migration participated in the observed topical anti-inflammatory activity.

Although a crucial role for leukotrienes has been suggested in some animal models of inflammation (Henderson 1994; Byrum et al 1997) and inhibitors of leukotriene synthesis have therapeutic potential in some inflammatory diseases such as asthma or psoriasis, selective inhibition of 5-LOX does not seem to be effective in experimental arthritis (Nickerson-Nutter & Medvedeff 1996). Therefore, compounds with additional anti-inflammatory mechanisms may achieve a better control of inflammation. When lupeol was tested on arachidonic acid-induced oedema, a model responsive for 5-LOX, it significantly reduced oedema formation at the highest dose tested.

It is interesting in the context of anti-inflammatory drug design to investigate possible biochemical mechanisms underlying the potentially beneficial activities of natural compounds to establish their mode of action. With this purpose, lupeol was further tested on some macrophage functions.

We evaluated the in-vitro anti-inflammatory activity of lupeol in cellular systems generating cyclooxygenase and lipoxygenase metabolites. Mouse peritoneal macrophages was chosen for the test system as it had the advantage that cellular activation with calcium ionophore A23187 caused the generation of both COX-1 products (e.g. PGE_2) and lipoxygenase products (LTC_4), eicosanoids usually used to estimate the activity of prostaglandin-synthase and 5-LOX, respectively.

When tested in this system, lupeol dose-dependently inhibited PGE_2 production, but it did not exhibit any significant effect on LTC_4 release, which strongly suggested that this triterpene did not affect the 5-LOX pathway. In this regard, it was interesting to note that these in-vitro results correlated well with those obtained in the murine inflammation model in-vivo.

On the other hand, thioglycolate-elicited peritoneal macrophages secrete PGE_2 (COX-2), NO and cytokines. Identification of selective inhibitors of inflammatory mediator production by these cells may lead to advances in the control of inflammation, as it was demonstrated that induction of iNOS and COX-2 resulted in a great increase in the synthesis of NO and PGE_2, which contributed to the pathophysiology of different inflammatory processes. In this sense, we have shown that lupeol could prevent the biosynthesis of PGE_2 as observed by the reduction of this eicosanoid level in culture medium of lipopolysaccharide-stimulated macrophages, a favourable feature for an anti-inflammatory profile with less adverse gastric reactions due to its relative selectivity to the COX-2 enzyme.

Macrophages also play a central role in chronic synovial inflammation. After activation they are capable of synthesizing mediators such as PGE_2 and cytokines such as TNF-α and IL-1. In turn, these secretory products induce the production of a variety of enzymes, which initiate cartilage and bone destruction (Shore et al 1986; Hopkins 1991). Thus, inhibition of these cytokines would exert anti-inflammatory effects. In this work, we have studied the effect of lupeol on cytokine production. The observation that lupeol was able to decrease TNFα and IL-1β release to some extent would agree with the previously reported inhibitory activity on adjuvant arthritis in rats.

In conclusion, this study has shown that lupeol was able to prevent the production of some pro-inflammatory mediators, which likely contributed to its anti-inflammatory activity. Recently, it has been suggested that the anti-inflammatory effects of some terpenes can be assigned, at least to a certain extent, to the inhibition of the activity of some transcription factors such as NF-κB, playing a key role in the regulated expression of several mediators including cytokines. In this context, we have reported the inhibitory action of the diterpene andalusol on NF-κB activity (De las Heras et al 1999). Indeed, this has been described for triterpenes (Suh et al 1998) and if this is the case of lupeol, it opens additional perspectives for the study of the therapeutic action of these molecules as anti-inflammatory agents.

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