Biochemical Pharmacology, Vol. 51, pp. 863–868, 1996.

ELSEVIER

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02249-X

SHORT COMMUNICATION

A Novel Diterpenoid Labdane from *Sideritis javalambrensis* Inhibits Eicosanoid Generation from Stimulated Macrophages But Enhances Arachidonate Release

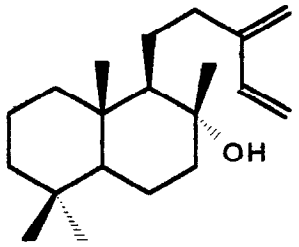
Linhua Pang, Beatriz de las Heras and J. R. S. Hoult†*
PHARMACOLOGY GROUP, KING'S COLLEGE LONDON, MANRESA ROAD, LONDON SW3 6LX, UK

ABSTRACT. The diterpenoid *ent*-8α-hydroxy-labda-13(16),14-dien ("labdane F2") was obtained from an anti-inflammatory extract of *Sideritis javalambrensis*. Labdane F2 inhibited prostaglandin E_2 generation in cultured mouse peritoneal macrophages, treated with zymosan, ionophore A23187, or arachidonic acid itself, and in J774 macrophage-like cells activated by bacterial lipopolysaccharide (LPS). The mechanism was investigated by prelabelling the macrophages with radiolabelled arachidonic acid or oleic acid, followed by cell activation in the presence or absence of nontoxic concentrations of labdane F2. Surprisingly, under those conditions in which reduced PGE_2 generation was observed, labdane F2 consistently enhanced the release of labelled fatty acid, in a manner similar to that displayed by thimerosal, a known acyl-CoA:lysolecithin transferase inhibitor. Labdane F2, therefore, appears to possess 2 mutually opposing actions on the eicosanoid system in macrophages: potentiation of delivery of substrate following cell activation, followed by inhibition of conversion of substrate to product. It was also found that nontoxic concentrations of labdane F2 reduced the expression of the inducible isoforms of cyclooxygenase and nitric oxide synthase in LPS-treated J774 cells. Thus, this anti-inflammatory diterpenoid labdane possesses a diverse array of effects impinging on enzyme pathways involved in eicosanoid generation and other inflammatory pathways in macrophages. BIOCHEM PHARMACOL 51;6:863–868, 1996.

KEY WORDS. Lamiaceae; diterpenoids; anti-inflammatory drugs; cyclo-oxygenase; nitric oxide synthase; transacylation; thimerosal

Recent studies on natural products obtained from plants from the genus *Sideritis* (Lamiaceae) have shown that they possess anti-inflammatory activity *in vivo* and *in vitro* [1–3]. We have found that the major diterpenoid product of *S. javalambrensis*, *ent*-8α-hydroxy-labda-13(16),14-dien ("labdane F2", structure I), inhibits the generation of cyclooxygenase and 5-lipoxygenase products of arachidonate metabolism in ionophore-stimulated rat peritoneal leukocytes [4]. A subsequent study showed that labdane F2 also inhibits its PGE_2‡ generation in cultured mouse peritoneal macrophages, although the precise mechanism and site of action of the inhibitor was not established [5].

We have, therefore, attempted to define the site which labdane F2 inhibits eicosanoid generation by testing its effects on mouse peritoneal macrophages and on mouse macrophage-like J774 cells. In some experiments, the macrophages were prelabelled with radioactive arachidonic or oleic acids to investigate whether or not F2 affects the release of unsaturated fatty acid precursors from membrane phospholipid.



I, labdane F2

MATERIALS AND METHODS

Isolation of Diterpenoids

The isolation from *S. javalambrensis* Pau of *ent*-8α-hydroxy-labda-13(16),14-dien has been described, as have the sources of materials [5]; thimerosal (ethylmercurithiosalicyclic acid) was obtained from Aldrich Chemical Company, Gillingham, U.K.

Prostaglandin E_2 Generation by Adherent Mouse Peritoneal Macrophages in Culture

Resident macrophages were isolated from male Swiss mice

* Affiliation: Department of Pharmacology, Complutense University, 28010 Madrid, Spain.
† Corresponding author.
‡ *Abbreviations:* A23187, calcium ionophore (calcimycin); COX-2, inducible isoform of cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; MTT, thiazolyl blue; NO, nitric oxide; iNOS, inducible isoform of nitric oxide synthase; PBS, phosphate buffered saline; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; PMA, phorbol myristate acetate.

Received 5 May 1995; accepted 9 November 1995.

and cultured in DMEM as before [6]. J774 cells obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, U.K.) were grown in DMEM containing 10% fetal calf serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL). Inhibitors and stimulants or appropriate volumes of their vehicles (usually 2 μL) were then added to 0.5×10^6 cells in 24-well plates in 1.0 mL medium, and incubation continued for a further 1 hr or 16 hr, depending on the stimulus. The inhibitors were usually added 30 min before the stimulant. The medium was carefully withdrawn from each well, centrifuged, decanted, and subjected to radioimmunoassay for PGE_2.

Labelling of Macrophages With Tritiated Oleic or Arachidonic Acids

Cells were plated out as above, but cultured overnight in the presence of 0.25 μCi/mL $^3H_8$-arachidonic acid (sp. act. 100 Ci/mmol, Dupont NEN) or 1.5 μCi/mL 9,10-^{3}H-oleic acid (10 Ci/mmol, Amersham, U.K.), after which they were washed twice and exposed to stimulants, with or without prior addition of test compound or vehicle, as indicated. The amounts of label were measured both in the medium and in the cells: thus, the medium was carefully removed and the cells solubilised in PBS containing 0.5% Triton X-100. Aliquots of 100 μL of medium and cell extract were then taken for scintillation counting, and the proportion of released fatty acid calculated as percentage of the total present.

MTT Assay for Cell Viability

Cell viability was measured by adding 50 μL 5 mg/ml MTT (thiazolyl blue) to cells in 24-well plates and incubating for 1 hr at 37°C. After removing the medium, 500 μL isopropanol was added to solubilize the blue-coloured tetrazolium and the plates were shaken for 5 min. Aliquots of 200 μL were transferred to 96-well plates and the OD_{550} values were read in a microplate reader. Viability was set as 100% in control cells.

Western Blotting for Nitric Oxide Synthase and Cyclooxygenase in J774 Cells

Expression of iNOS and COX-2 proteins was measured by Western blotting. After washing the cells with PBS, they were incubated for 5 min with an extraction buffer (0.9% NaCl, tris-HCl 20 mM, pH 7.6, triton X-100 0.1%, phenylmethylsulphonylfluoride 1 mM, leupeptin 0.01%) with gentle shaking. The cell extract was centrifuged (4000 *g*, 4°C, 10 min), and the protein concentration in the supernatant measured using the Folin-Ciocalteu reagent. Sufficient aliquots of sample were mixed 1:1 with sample buffer (tris-HCl 20 mM pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.025% bromophenol blue) and boiled for 5 min before electrophoresis in 7.5% sodium dodecyl sulphate-polyacrylamide gel (5 hr, 500V, 36 mA, 30 μg protein per track). Separated proteins were transferred to Biorad nitrocellulose membranes (2 hr, 40V) and the blot incubated for 1 hr with primary antibody (rabbit antisera to murine COX-2, Cayman, 1:1000, or to murine iNOS, gift of Dr. V. Riveros-Moreno, 1:1000). The blot was incubated with secondary antibody (sheep antirabbit IgG linked to horseradish peroxidase conjugate, Sigma, 1:2000) for 1 hr and, finally, incubated with ECL reagent (Amersham) for 1 min and exposed to Hyperfilm-ECL (Amersham).

RESULTS AND DISCUSSION

F2 Inhibits Enhanced Prostaglandin Generation in Stimulated Peritoneal Macrophages and J774 Cells

Similar to previous results [5], F2 dose-dependently reduced PGE_2 generation by mouse peritoneal macrophages cultured for 16 hr in the presence of calcium ionophore A23187, arachidonic acid, or zymosan (Fig. 1A).

We, therefore, tested the effects of F2 on PGE_2 generation by J774 cells. These cells are derived from mouse macrophages and resemble them in many (but not all) of their properties. For this reason, J774 cells are widely used as a convenient model for studying macrophage function. The results in Fig. 1B show that, although 1-hr incubation with A23187 and arachidonic acid did not cause enhanced PGE_2 release from the J774 cells, overnight culture with bacterial lipopolysaccharide caused a three-fold increase. This is because LPS causes the induction of cyclooxygenase-2 protein (see below), as well as the concurrent activation of PLA_2-dependent release of the substrate from membrane phospholipids. PGE_2 release from the LPS-treated cells was significantly inhibited by F2, whereas basal release in the control, A23187, and arachidonate-treated cells was unaffected (Fig. 1B).

These results do not clarify whether or not F2 acts directly on cyclo-oxygenase or on other processes involved in cellular activation resulting in eicosanoid generation. One possible site of action is at the level of phospholipase A_2, as activation of this enzyme is necessary for the release of arachidonic acid.

F2 Augments Unsaturated Fatty Acid Release in Peritoneal Macrophages and J774 Cells

To investigate the direct effects of F2 on phospholipase A_2, the cells were prelabelled with arachidonic acid as described in Methods, and then stimulated with A23187 or PMA. These substances are known to cause both arachidonate release and PGE_2 generation in mouse peritoneal macrophages [6–8], but their effects on the release of arachidonate in J774 cells have not been investigated. A23187 and PMA increased the release of arachidonic acid in mouse peritoneal macrophages (Fig. 2A, open columns), consistent with their known ability to activate arachidonate-specific cytosolic PLA_2 [9]. Surprisingly, it was found that the addition of F2 caused an increase in the amount of arachidonate released into the medium, both in unstimu-

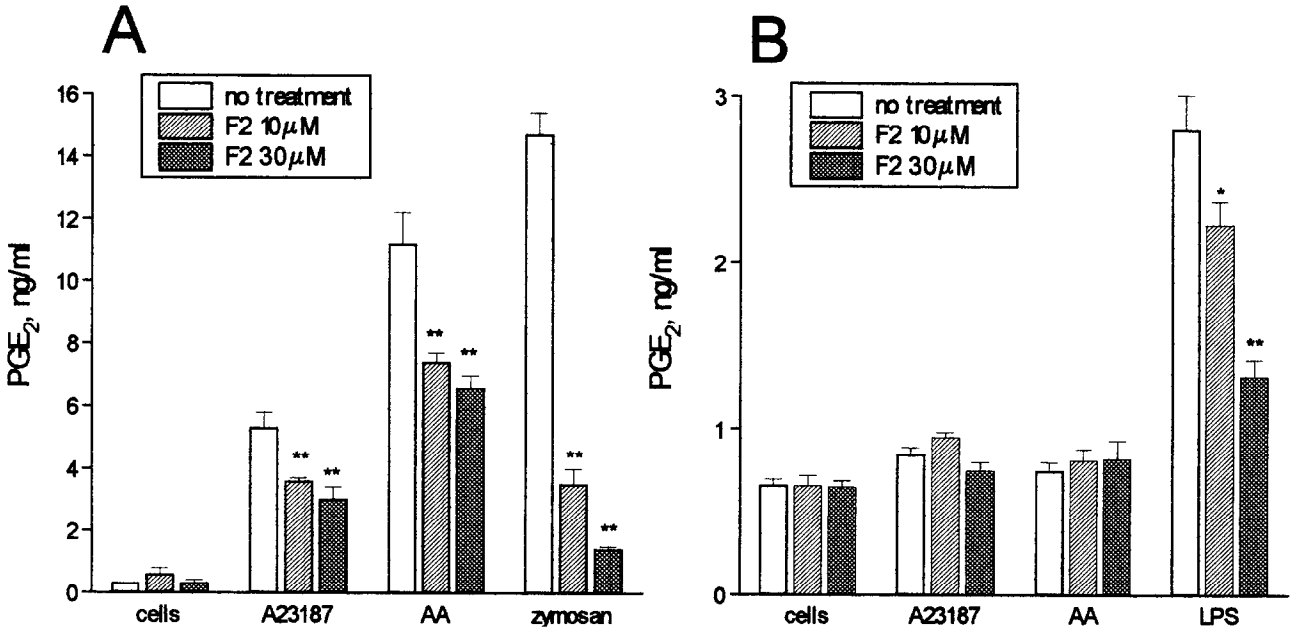


FIG. 1. Inhibition of PGE_2 generation by labdane F2 in mouse peritoneal macrophages (panel A) and J774 murine macrophage-like cells (panel B). The mouse peritoneal macrophages were incubated for 16 hr after adding 10^{-7} M calcium ionophore A23187, 10^{-6} M arachidonic acid or 20 μg/mL zymosan. The J774 cells were incubated for 1 hr after adding 10^{-5} M calcium ionophore A23187 or 10^{-5} M arachidonic acid, and 16 hr for the cells alone or after adding 500 ng/mL LPS. In all cases, labdane F2 was added 30 min before the stimulants. Results show mean ± SEM for duplicate determinations from 4 wells per treatment (mouse peritoneal macrophages) or 8 wells (J774 cells). The symbols * and ** indicate statistically significant reductions compared to vehicle-treated controls containing an equal volume of DMSO, $P < 0.05$, 0.01.

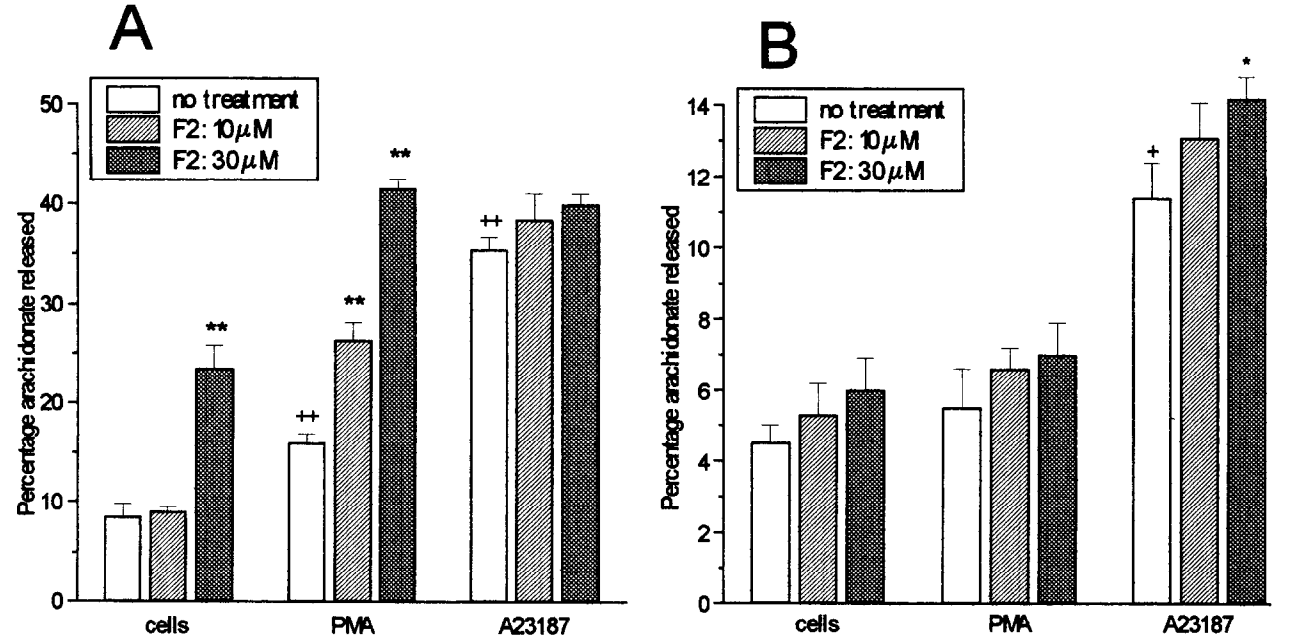


FIG. 2. Effect of pretreatment for 30 min with labdane F2 on the release of labelled arachidonate from mouse peritoneal macrophages (A) and J774 cells (B) under control conditions (addition of vehicle only) or after treatment for 2 hr with 10^{-5} M A23187 or 10^{-6} M PMA. Results show mean ± SEM for duplicate determinations from 4 wells per treatment (A) or 7 wells (B). The symbols $^{+},^{++}$ indicate statistically significant increases compared to vehicle-treated controls. $P < 0.05$, 0.01, and *,** indicate statistically significant increases after treatment with F2 compared to vehicle alone, $P < 0.05$, 0.01, respectively.

TABLE 1. Effect of thimerosal on arachidonate release from J774 cells

Treatment	% arachidonate release control	% arachidonate release + thimerosal, 1×10^5 M	% arachidonate release + thimerosal, 3×10^5 M
No addition	4.5 ± 0.5	7.4 ± 1.1*	21.4 ± 2.1†
+ PMA 10^{-6}M	5.5 ± 1.1	8.4 ± 0.7*	16.9 ± 1.2†
+ A23187 10^{-5}M	11.4 ± 1.0	16.3 ± 1.5*	17.2 ± 2.2*

Thimerosal was added 30 min prior to adding the stimulants at the concentrations indicated, followed by a further 2-hr incubation. Results show mean values from 7 wells for each treatment, and the symbols * and † indicate statistically significant increases (Students unpaired t-test), $P < 0.05$ and 0.001, respectively.

lated cells and in those treated with PMA and A23187, although the latter was not significant (Fig. 2A). However, in J774 cells, only A23187 caused release of arachidonic acid, although this was significantly increased by pretreatment with 3×10^{-5} M F2 (Fig. 2B). Labdane caused small, but nonsignificant, increases in arachidonate release in control and PMA-treated J774 cells.

One explanation for these results could be that F2 might also act to inhibit acyl-CoA: lysolecithin acyltransferase. This enzyme reinserts fatty acids into the *sn*-2 position of lysophospholipids, thus restoring the membrane fatty acid architecture of phospholipids (the so-called Lands cycle) [10, 11]. Thimerosal is known to inhibit this enzyme [12, 13]. Indeed, thimerosal also potentiated arachidonate release in J774 cells under basal and stimulated conditions (Table 1). However, the higher dose of thimerosal (3×10^{-5} M) was associated with considerable toxicity after 2-hr exposure, as was obvious from visual inspection of the cells.

Oleic acid may also be incorporated into the *sn*-2 position of membrane phospholipids, but is not released selectively by cytosolic PLA_2. However, it may be released by other PLA_2s such as the mammalian nonpancreatic 14 kDa enzymes [14, 15]. Figure 3 compares the release of preincorporated arachidonic acid and oleic acid from mouse peritoneal macrophages. Like arachidonate, the preincorporated oleic acid was released from the washed macrophages during 2-hr incubation, but this was not augmented by treatment of the cells with A23187 or PMA. On the other hand, F2 significantly increased the release of free oleic acid as it did for arachidonic acid (Fig. 3), as would be expected if the reacylation reaction was impaired.

F2 is Not Cytotoxic to J774 Cells but Protects Against NO Toxicity by Reducing LPS-Induced NO Generation

It was previously shown that F2 at concentrations up to 3×10^{-5} was not cytotoxic to neutrophils or macrophages [5],

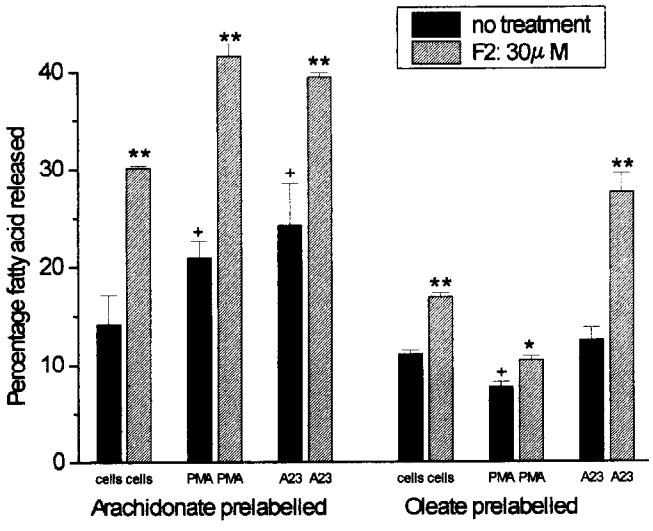


FIG. 3. Effect of A23187 and PMA on the release of preincorporated arachidonic acid and oleic acid from mouse peritoneal macrophages in the presence and absence of labdane F2 (30-min pretreatment, 2-hr incubation). Results show mean ± SEM for duplicate determinations from 8 wells per treatment, derived from 2 separate experiments. Symbol + indicates statistically significant increase compared to vehicle-treated control, $P < 0.05$; *, indicate statistically significant increases after treatment with F2 compared to no treatment, $P < 0.05$, $P < 0.01$, respectively.**

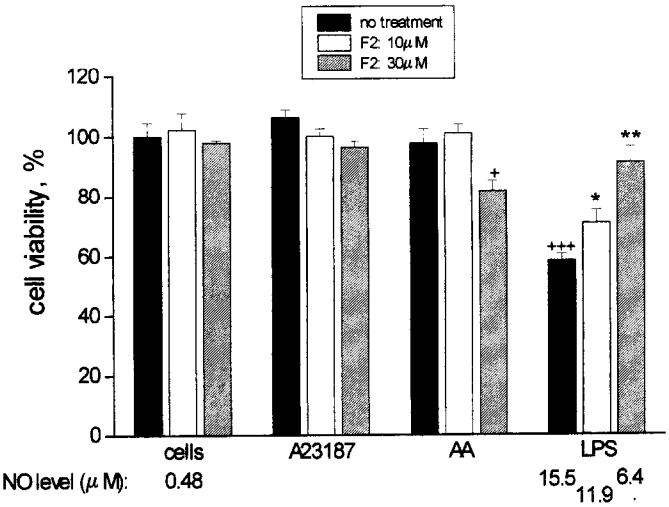


FIG. 4. Effect of labdane F2 and the treatment with A23187, AA, and LPS on the viability of J774 cells. In all cases, labdane was added 30 min before the cellular stimulant. AA and A23187 were added for 2 hr, whereas exposure to LPS or cells alone was 16 hr. Amounts of NO released into the culture medium are shown under the columns and were measured using the Griess reagent. NO levels were not measured in J774 cells treated with A23187 or AA because these treatments do not activate the L-arginine:NO system under these conditions. Results show mean ± SEM for 4 tests and symbols +,+++ indicate statistically significant decrease compared to vehicle-treated control, $P < 0.05$, $P < 0.001$, whereas *, indicate statistically significant increases in viability caused by preincubation with F2, $P < 0.05$, $P < 0.01$.**

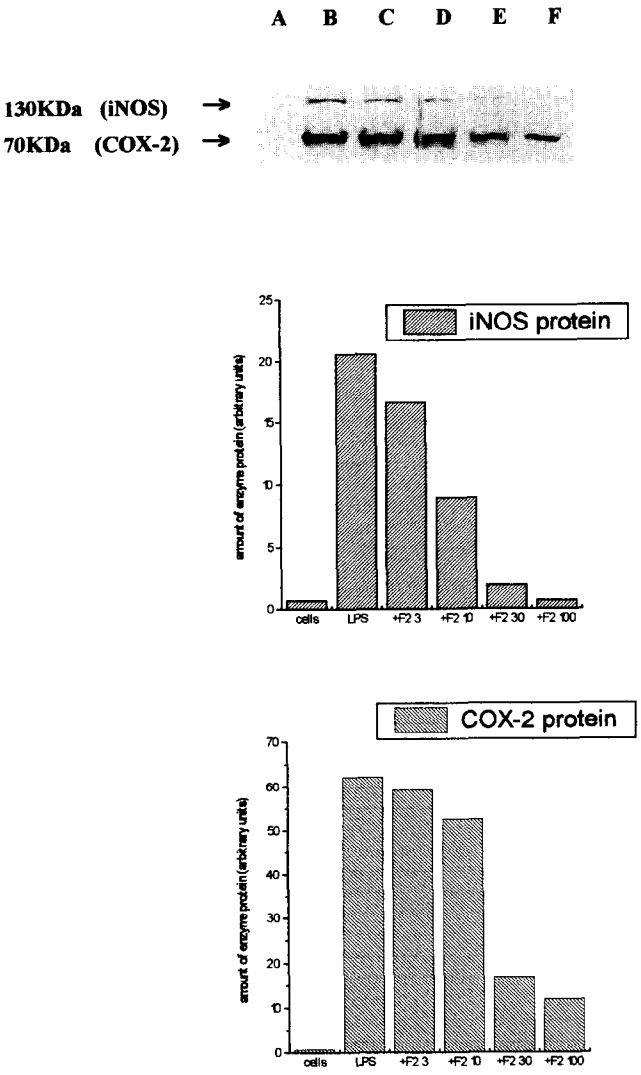


FIG. 5. Effect of labdane F2 on expression of LPS-induced iNOS and COX-2 enzyme proteins. Top part shows immunoelectrophoretic identification of the two enzymes in extracts from J774 cells treated as follows: A, cells alone; B, 16-hr treatment with 500 ng/mL LPS; C to F, as B but pretreated for 30 min with labdane F2 at concentrations (μM) of 3, 10, 30, and 100. The bottom panels show the amounts of proteins as measured using a densitometer scanner and MCID software (Imaging Research Inc., Canada), expressed as arbitrary units.

although 10×10^{-5} M was weakly cytotoxic and mildly haemolytic. This was checked in the present experiments using J774 cells incubated in the presence of 1×10^{-5} M and 3×10^{-5} M F2, and tested for viability using the MTT assay. This method measures mitochondrial respiratory integrity and has been widely used in studies of cell viability [16]. Figure 4 shows that treatment of J774 cells with A23187 and arachidonate for 2 hr did not adversely affect their viability, and that this was not altered by F2 at the concentrations tested, except for a small but significant loss of viability in cells treated with arachidonate + 3×10^{-5} M F2.

In contrast, 16-hr incubation with bacterial lipolysaccharide significantly reduced macrophage viability. However, F2 offered protection that was dose-related (Fig. 4). We attribute this to the ability of F2 to inhibit the generation by these cells of NO. The figures below the columns in Fig. 4 show that the diterpenoid labdane significantly reduced NO generation in a dose-dependent manner, and this has subsequently been confirmed (L. Pang and J R S Hoult, unpublished experiments).

Treatment of J774 cells with LPS is known to cause the induction of the isoforms of nitric oxide synthase and cyclooxygenase (iNOS and COX-2, respectively), thought to be responsible for the large increases in generation of NO and PGE_2. In view of the ability of F2 to inhibit production of both these mediators in LPS-treated J774 cells, we investigated its effects on iNOS and COX-2 expression using Western blotting. This confirmed the induction of iNOS and COX-2 enzymes by LPS, but also showed that F2 reduced their expression (Fig. 5). This could explain why labdane F2 reduces the generation of PGE_2 and NO under the present experimental conditions.

In summary, we have found that the anti-inflammatory plant-derived labdane F2 inhibits prostaglandin generation in stimulated macrophages. However, it also enhances the release from membrane phospholipids of unsaturated fatty acids such as oleic acid and arachidonic acid. This could be due to inhibition of acyl-CoA:lysolecithin transferase, although this needs to be tested directly. However, there is no structural resemblance between labdane F2 and thimerosal, a known inhibitor of acyl-CoA:lysolecithin transferase. An alternative possibility is that the highly lipophilic labdane F2 might interpolate into biomembranes in a manner sufficient to alter the activity of enzymes embedded in them. Whatever the precise explanation, we have shown that F2 appears to possess 2 mutually opposing actions on the eicosanoid system in macrophages: potentiation of delivery of substrate following cell activation, followed by inhibition of conversion of substrate to product. Labdane also prevents the induction of both iNOS and COX-2 by LPS in J774 cells without apparent cell toxicity. This might be the primary explanation for its inhibitory effects on mediator production and requires further analysis. Thus, overall, it is clear that this diterpenoid possesses a unique and diverse array of properties that lead to the alteration of proinflammatory enzyme pathways in macrophages.

L. Pang thanks the World Health Organisation for a Fellowship. Partially supported by the CRF (University of London) and Complutense University (Madrid).

References

1. Villar A, Gasco MA and Alcaraz MJ, Anti-inflammatory and anti-ulcer properties of hypolaetin-8-glucoside, a novel plant flavonoid. *J Pharm Pharmacol* **36:** 820–823, 1984.
2. Ferrándiz ML and Alcaraz MJ, Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. *Agents Actions* **32:** 283–288, 1991.

3. de las Heras B, Vivas JM and Villar A, Anti-inflammatory activity of *Sideritis javalambrensis*. *Planta Med* **56:** 658–659, 1990.
4. de las Heras B, Villar A, Vivas JM and Hoult JRS, Novel anti-inflammatory plant labdanes: comparison of *in vitro* properties with aspirin and indomethacin. *Agents Actions* **41:** 114–117, 1994.
5. de las Heras B and Hoult JRS, Non cytotoxic inhibition of macrophage eicosanoid biosynthesis and effects on leukocyte functions of two novel anti-inflammatory plant diterpenoids. *Planta Med* **61:** 33–36, 1995.
6. Bonney RJ, Wightman PD, Dahlgren ME, Davies P, Kuehl FA and Humes JL, Effect of RNA and protein synthesis inhibitors on the release of inflammatory mediators by macrophages responding to phorbol myristate acetate. *Biochim Biophys Acta* **633:** 410–421, 1980.
7. Tripp CS, Mahoney M and Needleman P, Calcium ionophore enables soluble agonists to stimulate macrophage 5-lipoxygenase. *J Biol Chem* **260:** 5895–5898, 1985.
8. Aderem AA and Cohn ZA, Bacterial lipopolysaccharides modify signal transduction in the arachidonic acid signal cascade in macrophages. In: *Biochemistry of Macrophages*, pp. 196–210. John Wiley, Chichester, 1986.
9. Lin LL, Lin AY and Knopf JL, Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc Natl Acad Sci USA* **89:** 6147–6151, 1992.
10. Lands WEM, Metabolism of glycerolipids II. The enzymatic acylation of lysolecithins. *J Biol Chem* **235:** 2233–2237, 1960.
11. Magargal WW, Dickinson ES and Slakey LL, Distribution of membrane marker enzymes in cultured arterial endothelial and smooth muscle cells. The subcellular location of oleoyl: CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase. *J Biol Chem* **253:** 8311–8318, 1978.
12. Goppelt-Strübe M, Korner CF, Hausmann G, Gemsa D and Resch K, Control of prostanoid synthesis: role of reincorporation of released precursor fatty acids. *Prostaglandins* **32:** 373–386, 1986.
13. Förstermann U, Goppelt-Strübe M, Fröhlich JC and Busse R, Inhibitors of acyl-coenzyme A:lysolecithin acyltransferase activate the production of endothelium-derived vascular relaxing factor. *J Pharmacol Exp Ther* **238:** 352–359, 1986.
14. Glaser KB, Mobilio D, Chang JY and Senko N, Phospholipase A_2 enzymes: regulation and inhibition. *Trends Pharmacol Sci* **14:** 92–98, 1993.
15. Seilhamer JJ, Pruzanski W, Vadas P, Plant S, Miller JA, Kloss J and Johnson LK, Cloning and recombinant expression of phospholipase A_2 present in rheumatoid arthritic synovial fluid. *J Biol Chem* **264:** 5335–5338, 1989.
16. Mossmann T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:** 55–63, 1983.