Prostaglandin H-Synthase-2 Is the Main Enzyme Involved in the Biosynthesis of Octadecanoids from Linoleic Acid in Human Dermal Fibroblasts Stimulated with Interleukin-1β

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This study was focused on the characterization of the metabolism of linoleic acid by human dermal fibroblasts and the effect of interleukin-1 on the biosynthesis of octadecanoids. Dermal fibroblasts untreated and treated with recombinant IL-1β were incubated with exogenous labeled linoleic acid. A combination of high performance liquid chromatography and gas chromatography–mass spectrometry was used as the analytic technique. We found that dermal fibroblasts convert linoleic acid mainly into 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid (13-HODE) and 9-hydroxy-10-*trans*,12-*cis*-octadecadienoic acid (9-HODE), 13(S)-HODE and 9(R)-HODE being the predominant enantiomers. IL-1β increased the formation of both 13-HODE and 9-HODE in a concentration-dependent manner with similar EC_{50} values as for prostanoid formation. This effect of IL-1β on HODEs formation was concomitant with the expression of prostaglandin H-synthase-2. Formation of octadecanoids was inhibited in a concentration-dependent manner by acetylsalicylic acid and indomethacin. Dexamethasone, actinomycin D, and cycloheximide abolished the effect of IL-1β on HODEs biosynthesis. Octadecanoid biosynthetic activity was associated with the microsomal fraction. Dermal fibroblasts incorporated [^{14}C]-9-HODE and [^{14}C]-13-HODE into phospholipids, mainly into phosphatidylcholine. IL-1β increased significantly the esterification of 13-HODE in all glycerophospholipids, the major increase being observed in phosphatidylinositol. These results indicate that prostaglandin H-synthase-2 is the enzyme responsible for the increase in the ability to form HODEs of dermal fibroblasts stimulated with IL-1β. ***Key words: hydroxy-octadecadienoic acid/cytokine/cyclooxygenase/phospholipid. J Invest Dermatol 107:726–732, 1996***

Linoleic acid (LA) is an essential *cis*-polyunsaturated fatty acid and the precursor of 13- and 9-hydroperoxy-octadecadienoic (HPODE) acids, which in turn are reduced to the corresponding hydroxy-octadecadienoic (HODE) acids, these being the major oxygenated metabolites of LA produced by cells. HPODEs and HODEs have been found in high amounts in atherosclerotic (Künn, 1992) and psoriatic lesions (Camp *et al.*,1983; Baer *et al*, 1990, 1991) and have several biologic activities involved in the inflammatory response (Buchanan *et al*, 1985; Yamaja Setty *et al*, 1987; Iversen *et al*, 1991; Ku *et al*, 1992).

The site of oxygen insertion on LA is primarily at the C9 or C13 and is largely dependent on the cell type. In general, enzymatic oxidation of LA may involve both prostaglandin H synthase (PGHS) and 15-lipoxygenase (15-LO) activities (Hamberg and Samuelsson, 1967; Reinaud *et al*, 1989), which are differentially expressed. Biosynthesis of HPODEs and HODEs has been reported to occur in several cells including vascular (Buchanan *et al*, 1985; Camacho *et al*, 1995) and epidermal (Nugteren and Kivits, 1987) cells, platelets (Daret *et al*, 1989), and leukocytes (Reinaud *et al*, 1989), but the precise biosynthetic pathway is often not well established.

A characteristic feature in the immune response is the cooperation between cytokines and lipid mediators in the regulation of inflammatory and proliferative responses. Interleukin-1 (IL-1) is a pleiotropic cytokine that plays a central role in inflammation. IL-1 induces the expression of adhesion molecules and secondary cytokines which, in turn, induce acute and chronic inflammatory changes (Dinarello, 1991). IL-1 also stimulates the release of prostaglandins and promotes the expression of prostaglandin H-synthase (PGHS) (Raz *et al*, 1988; Maier *et al*, 1990). Two PGHS isoenzymes encoded by different genes have been characterized: PGHS-1 is expressed in a constitutive manner and PGHS-2 is the inducible isoenzyme by mitogens, which is overexpressed in many inflammatory processes (Smith, 1992; Hla *et al*, 1993).

Dermal fibroblasts are able to release factors that may control the

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Abbreviations: ASA, acetylsalicylic acid; PGHS, prostaglandin H-synthase; EC_{50}: one-half maximum effective concentration; EI, electron impact; GC-MS, gas chromatography-mass spectrometry; 13-HODE, 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid; 9-HODE, 9-hydroxy-10-*trans*,12-*cis*-octadecadienoic acid; LA, linoleic acid; LO, lipoxygenase; SP-HPLC, straight phase-HPLC.

activity of the epidermis, such as cytokines (Larson *et al*, 1989; Kupper, 1990), and also eicosanoids such as prostaglandin E_2 (PGE_2) or 15-hydroxyeicosatetraenoic acid (Mayer *et al*. 1984). We reported that IL-1 increased the ability of dermal fibroblasts to form 15-hydroxyeicosatetraenoic acid and that this was due to an increase of PGHS activity rather than to an enhanced 15-LO activity (Godessart *et al*, 1994). Nevertheless, the expression of the two isoenzymes of the PGHS was not evaluated at that time. Because IL-1 is a central mediator in the inflammatory response on skin and because HPODEs and HODEs play a modulatory role in inflammatory and proliferative responses, we wished to investigate the metabolism of LA in dermal fibroblasts, the effect of IL-1 on it, and the enzyme involved in that effect.

MATERIALS AND METHODS

Fibroblast Isolation and Culture Cells were isolated from skin samples of breast and abdomen of women who had undergone plastic surgery. Dermis and epidermis were separated according to the technique described by Liu and Karasek (1978). The fragments of the dermis were then incubated overnight in complete culture medium [Dulbecco's modified Eagle's medium (DMEM)] containing 10% fetal bovine serum (Bio-Whittaker, Walkersville, MD) and 0.5 mg collagenase Type Ia per ml (Boehringer Mannheim S.A., Barcelona, Spain). The suspension was filtered through sterile gauze, collected by centrifugation, and seeded in 75-cm^2 culture flasks at a density of 10^6 cells/flask. When the culture reached confluence, cells were released with 0.2% trypsin and split at a ratio of 1:3. Cells were detached and seeded in six-well plates at a density of 10^5 cells per ml and used at confluence ($6–7.10^5$ cells/well). All experiments were performed with cells between passages 5 to 7.

Incubations of Fibroblasts with [^{14}C]LA Confluent cells in six-well plates were incubated at 37°C in the presence of 0.4 ml medium containing 10 mM HEPES, 2 mM $CaCl_2$, and the indicated concentration of [^{14}C]LA (50–53 mCi/mmol, DuPont NEN, Boston, MA) in 5 μl of ethanol. After the indicated periods of time, the reactions were stopped by addition of 1 N HCl to yield a pH of 3 followed by one volume of cold methanol. Samples were kept at −80°C until analysis.

Nonenzymatic formation of HODEs was estimated by incubating [^{14}C]LA with boiled cells.

Analysis of Octadecanoids Straight-phase-high performance liquid chromatography (SP-HPLC), gas chromatography-mass spectrometry (GC-MS), and chiral analysis were performed essentially as described previously (Camacho *et al*, 1995).

Incubations with IL-1β Fibroblasts were incubated for the indicated times with 1 ml of DMEM containing 1% (vol/vol) fetal bovine serum and 0–20 U human recombinant IL-1β (Boehringer Manheim, Indianapolis, IN) per ml. Medium was removed, and the cells were washed with 2 ml of 0.1 M phosphate-buffered saline (0.16 M NaCl, pH 7.4). Cells were then incubated with [^{14}C]LA, as described, or disrupted for RNA extraction.

PGHS-1 and -2 Specific mRNA Analysis Total RNA was isolated by phenol–chloroform extraction and isopropanol precipitation according to the protocol described by Chomczynski and Sacchi (1987) and quantitated spectrophotometrically by absorption at 260 and 280 nm. The specific mRNA levels were estimated by means of a quantitative reverse transcriptase-polymerase chain reaction protocol: briefly, 1 μg of total RNA was reverse transcribed into cDNA by incubation with 50 U of murine leukemia virus reverse transcriptase in a reaction buffer containing 10 mM Tris(hydroxymethyl)-aminomethane-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 2.5 μM random hexamers, 20 U of Rnasin (GeneAmp RNA polymerase chain reaction kit, Perkin-Elmer, Branchburg, NJ), and 1 mM deoxynucleoside triphosphates (Epicentre Technologies, Madison, WI) in a final volume of 20 μl. The reaction mixture was incubated 30 min at 42°C, and the reaction was stopped by heating 5 min at 99°C and then 5 min at 5°C.

The primers used for PGHS-1 and PGHS-2 were 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′(sense) and 5′-GTGCATCAACACAGGCGCCTCTTC-3′ (anti-sense) and 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ (sense) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (anti-sense), respectively (Hla and Neilson, 1992). The RNA for glyceraldehyde-3-phosphate-dehydrogenase was amplified and used as the internal control. The sense and anti-sense primers used for glyceraldehyde-3-phosphate-dehydrogenase were 5′-CCACCCATGGCAAATTCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′, respectively (Maier *et al*, 1990). Polymerase chain reaction was carried out in a DNA Thermal Cycler 480 (Perkin-Elmer) with a reaction mixture (100 μl) containing 10 mM Tris(hydroxymethyl)-aminomethane-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 1 μM sense or anti-sense primers, 2.5 U *Taq* polymerase (Perkin-Elmer), and 4 μCi of [^{3}H]deoxycytidine triphosphate (48–71 Ci/mmol, Amersham Ibérica, Madrid, Spain). Serial half-dilutions of the cDNA were done in order to prove the linearity. Twenty seven cycles were performed as follows for all the samples: 1.5 min at 94°C, 1.5 min at 58°C, and 1.5 min at 72°C. The products of the amplification were separated in a 1.5% low melting point agarose gel (GIBCO BRL, Paisley, Scotland) containing ethidium bromide (0.5 μg per ml, Perkin Elmer). To remove excess [^{3}H]deoxycytidine triphosphate and to avoid excessive background, when the loading dye had migrated half way through the gel, the buffer was replaced by a fresh one. The bands were visualized, cut out under ultraviolet (UV) light using a circular template, and melted by heating to 70°C. Complete digestion of gel pieces was performed by adding 1 ml of the tissue solubilizer NCS-II (Amersham Ibérica) and was left in a water bath at 50°C for 1 h. Afterward, 10 ml of scintillation cocktail (Ecoscint H, National Diagnostics, Atlanta, GA) were added, and radioactivity was monitored in a β-counter (LS-3800, Beckman, San Ramón, CA) and normalized with respect to glyceraldehyde-3-phosphate-dehydrogenase.

Western Blot Analysis Western blot analysis of PGHS-1 and -2 in fibroblasts treated with 10 U IL-1β per ml for the indicated periods of time was performed as described previously (Camacho *et al*, 1995). PGHS-1 isolated from ram seminal vesicles and PGHS-2 purified from sheep placenta were obtained from Cayman Chemical Co (Ann Arbor, MI). Rabbit polyclonal antiserums against PGHS-1 (PG 20) and PGHS-2 (PG 27) were from Oxford Biomedical Research, Inc. (Oxford, MI).

Determination of PGHS Activity After cells had been exposed to 10 U IL-1β per ml for the desired period of time, they were incubated at 37°C in the presence of 25 μM [^{14}C]arachidonic acid ([^{14}C]-AA, 55–58 mCi/mmol, Amersham Ibérica) in 5 μl of ethanol. After 15 min the reactions were stopped by addition of a previously measured volume of 1 N HCl to yield pH 3 followed by one volume of cold methanol. Samples were kept at −80°C until HPLC analysis. Analysis of prostanoids was performed as described previously (Solá *et al*, 1992).

Effect of PGHS Inhibitors on LA Metabolism Cells were preincubated in the presence or in the absence of 10 U IL-1β per ml for 24 h. Medium was removed, and cells were washed and treated with 1 ml of DMEM containing the indicated concentrations of indomethacin or acetylsalicylic acid (ASA) dissolved in ethanol [final ethanol concentration, 0.1% (vol/vol)] for 10 min. ASA-treated cells were then washed twice with phosphate-buffered saline and incubated with [^{14}C]LA as described above.

Effect of the Inhibition of Protein Synthesis on the Action of IL-1β on LA Metabolism The cells were incubated for 8 h with 10 U IL-1β per ml in the presence or in the absence of 3 μg cycloheximide per ml or 1 μM actinomycin D (Sigma, St Louis, MO). Fibroblasts were then incubated with 50 μM [^{14}C]LA for 15 min as described above.

Effect of Dexamethasone on the Action of IL-1 on [^{14}C]LA Metabolism Dermal fibroblasts were incubated for 8 h in the absence and in the presence of 10 U IL-1_β per ml with or without 10 nM dexamethasone (Sigma). Cells were then incubated with 50 μM [^{14}C]LA for 15 min, and the production of octadecanoids was evaluated.

Cell Fractionation Cells grown in 150-cm^2 culture flasks were incubated for 24 h with 10 ml of DMEM plus 1% (vol/vol) fetal bovine serum in the presence or in the absence of 10 U IL-1β per ml. Afterward, cells were washed with phosphate-buffered saline and detached with 5 ml of trypsin solution [0.1% (wt/vol) in phosphate-buffered saline]. Cells were washed twice and centrifuged at 250 × *g* for 10 min, and the pellet was resuspended in 1 ml of 10 mM Tris(hydroxymethyl)-aminomethane-HCl, pH 7.2, containing 0.1% (wt/vol) soybean trypsin inhibitor and protease inhibitors (1 mM sodium metabisulfite, 1 mM benzamidine, 1 mM phenylmethylsulfonylfluoride, 1 mM ethylenediamine tetraacetic acid, and 1 mM ethyleneglycol bis(b-aminoethylether)-N,N,N′,N′-tetraacetic acid). Cells were then disrupted mechanically with a Potter-Elvehjem homogenizer placed in an ice-water bath. Unfractionated cells were eliminated by centrifugation at 700 × *g* for 10 min, and the resulting supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was collected, and the pellet corresponding to the microsomal fraction was resuspended in the buffer mentioned above. The protein content, measured by the method of Lowry (Protein Assay Kit, Sigma), was adjusted to 1.67 mg per ml. Aliquots of 0.6 ml of cell fractions were then incubated with 50 μM [^{14}C]LA plus 2 mM phenol at 37°C for 10 min. Reactions were stopped and octadecanoids were extracted and analyzed.

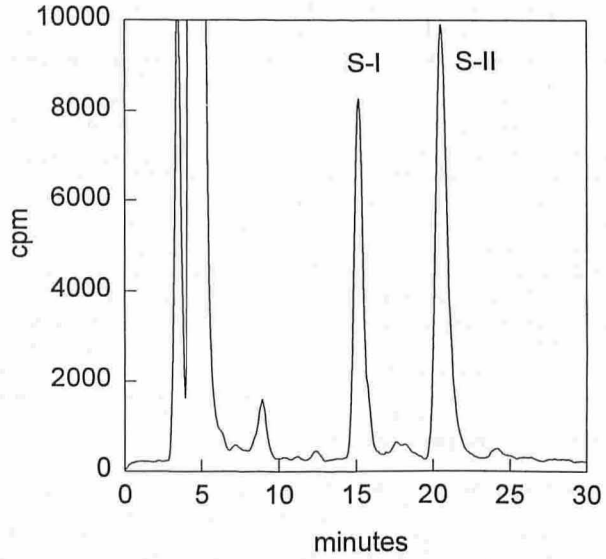

Figure 1. Fibroblasts produced two main products, S-I and S-II, with a chromatographic retention time identical to that of authentic 13-HODE and 9-HODE, respectively. Representative SP-HPLC chromatogram from a sample of dermal fibroblasts incubated with 50 μM [^{14}C]LA for 15 min. Column: Ultrasphere-Si 4 × 250 mm, 5 μm, Beckman; solvent: diethyl ether/*n*-hexane/acetic acid 30:70:0.1; flow rate: 1 ml/minute (Camacho *et al*, 1995).

Esterification of 13-HODE and 9-HODE into Phospholipids [^{14}C]13-HPODE was obtained by incubating [^{14}C]LA with soybean lipoxygenase (Sigma). [^{14}C]13-HODE was purified after reduction of [^{14}C]13-HPODE with $NaBH_4$ by collecting the corresponding peak after SP-HPLC. [^{14}C]9-HPODE was obtained by incubating [^{14}C]LA with tomato fruit homogenate according to the method described by Matthew *et al* (1977). [^{14}C]9-HPODE was then reduced with $NaBH_4$, and [^{14}C]9-HODE was purified by SP-HPLC. Cells grown in 75-cm^2 culture flasks, untreated or treated with IL-1β for 24 h, were incubated in 2.5 ml of DMEM containing 2% (vol/vol) BFS and 2.5 μM [^{14}C]13-HODE or [^{14}C]9-HODE for 3 h at 37°C. Cells were washed with DMEM twice, after which 3 ml of 2% acetic acid in methanol and 3 ml of water were added to the flasks, cells were scraped off the flask, and lipids were extracted according to the method described by Bligh and Dyer (1959). Extracts were dried under a N_2 stream, and the residues were redissolved in 35 μl of methanol/chloroform (1:2) and seeded in silica gel 150A thin-layer chromatography plates (LK5D, Whatman, Maidstone, England). Chromatography was carried out as follows: a mixture of ethyl acetate:isooctane:acetic acid:water (55:25:10:50) was vigorously shaken and allowed to equilibrate for 15 min at room temperature, after which the upper phase was used as eluent. The phospholipid fraction, remaining in the origin of the plate, was scraped off and extracted three times with 400 μl of methanol:chloroform (1:2). Extracts were dried under an N_2 stream, redissolved in 35 μl of methanol:chloroform (1:2), and rechromatographed according to the method of Fine and Sprecher (1982). Plates were autoradiographed at −40°C for 72 h (Autoradiography film Curix RP2, developer G138 from Agfa-Gevaert, Barcelona, Spain), and each spot was identified by comigration with authentic standards. Spots were scraped off, and the radioactivity was measured in a liquid scintillation counter using Biogreen II as scintillation cocktail (Reactivos Scharlau, Barcelona, Spain). Authentic ^{14}C-labeled standards of phosphatidylcholine, phosphatidyl-ethanolamine, phosphatidylinositol, phosphatidyl-serine, and sphingomyelin were supplied by Amersham Ibérica.

RESULTS

The Main Octadecanoids Formed by Dermal Fibroblasts Were 9(R)-HODE and 13(S)-HODE The analysis of samples from incubations of fibroblasts with [^{14}C]LA by SP-HPLC revealed the presence of two major peaks (peak S-I and S-II **Fig 1**) that had the same retention times as authentic standards of 13-HODE and 9-HODE. Occasionally, three minor additional peaks were observed.

To further characterize the major compounds formed by dermal fibroblasts from LA, we collected and derivatized the material eluting as the 13-HODE and 9-HODE fractions from SP-HPLC (peaks S-I and SII, respectively). Each purified compound was derivatized as the Me_3Si ether methyl ester and subsequently analyzed by GC-MS. The electron impact (EI) mass spectrum of peak S-I **(Fig 2)** was essentially identical to that previously reported for 13-HODE (Glasgow and Eling, 1990; Camacho *et al*, 1995), and the GC retention time and EI mass spectrum were identical to those of authentic 13-HODE. A molecular ion was detected at mass/charge (m/z) 382 (M^+). Additional informative ions were present at m/z 311 [M^+-71, loss of $(CH_2)_4CH_3$] and 225 [M^+-157, loss of $(CH_2)_7COOCH_3$]. Similar ions occurred in the S-II spectrum **(Fig 2)**, although the relative intensities were different; the spectrum of this compound was identical to those reported for 9-HODE (Glasgow and Eling, 1990; Camacho *et al*, 1995) and to authentic 9-HODE, GC retention time was also similar to that of authentic 9-HODE. To determine the location of the hydroxyl group, methyl esters were catalytically hydrogenated before trimethysilylether derivatization. The EI mass spectrum of saturated S-I **(Fig 2)** contained major fragment ions at m/z 315 [M^+-71, loss of $(CH_2)_4CH_3$] and 173 [M^+-213, loss of $(CH_2)_{11}COOCH_3$]. The fragment ion at m/z 315 suggested the location of the hydroxyl group at C-13 and cleavage of the bond between C-13 and C-14. The ion at m/z 173 also indicated the presence of a hydroxyl group at C-13 and represents cleavage of the carbon bond between C-12 and C-13. The EI mass spectrum of hydrogenated S-I was essentially identical to those reported for hydrogenated 13-HODE (Glasgow and Eling, 1990; Camacho *et al* 1995) and authentic 13-HODE. The UV spectrum of peak S-I was identical to that of authentic 13-HODE and had a λ_{max} value of 234–235, which revealed the presence of a conjugated *cis,trans*-hydroxy diene system (Ingram and Brash, 1988). HPLC, GC-MS, and UV analysis indicates that peak S-I corresponded to 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid (13-HODE).

The EI mass spectrum of saturated S-II **(Fig 2)** contained major fragment ions at m/z 259 [M^+-127, loss of $(CH_2)_8CH_3$] and 229

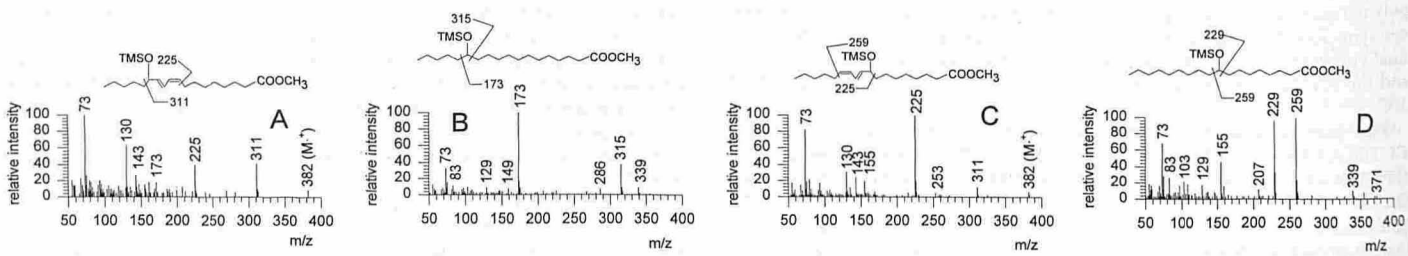

Figure 2. GC-MS analysis shows that the identity of S-I and S-II was 13-HODE and 9-HODE, respectively. Each purified compound was derivatized as the trimethylsilyl ether methyl ester and subsequently analyzed by GC-MS (S-I,*panel A*; and S-II, *panel C*). Each purified compound was also derivatized as methyl ester and subsequently catalytically hydrogenated before trimethylsilyl derivatization. EI mass spectra from GC-MS analysis of saturated compounds are shown in *panels B* and *D*. The schemes of fragmentation show the origin of the most characteristic fragments.

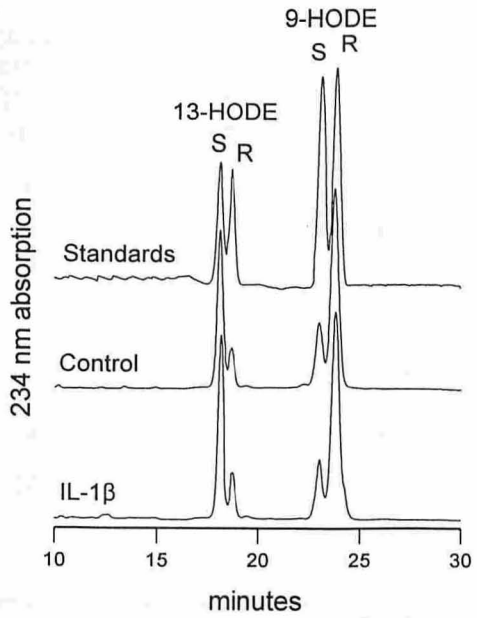

Figure 3. 9(R)-HODE and 13(S)-HODE were the main enantiomers formed by dermal fibroblasts. These are representative chiral phase chromatograms of the methyl esters of standards and samples from untreated and IL-1β–treated cells (10 U per ml for 24 h) incubated with 25 μM [^{14}C]LA for 10 min. Ratios of enantiomers are shown in the text.

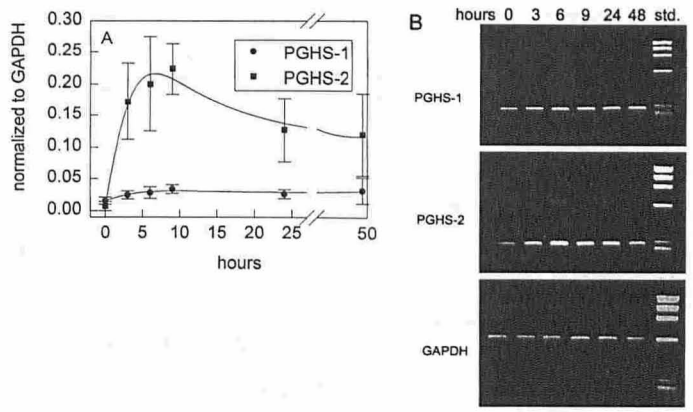

Figure 4. IL-1β mainly induced transcription of PGHS-2 gene. *(A)* Specific mRNA for PGHS-1 and -2 expression normalized to glyceraldehyde-3-phosphate-dehydrogenase in human dermal fibroblasts as a function of time of treatment with 10 U IL-1β per ml; data are the mean of two separate experiments, quoted by individual values; and *(B)* representative gel photographs from polymerase chain reaction samples corresponding to the indicated periods of time of treatment with 10 U IL-1β per ml.

[M^+-157, loss of $(CH_2)_7COOCH_3$]. The major fragment ion at m/z 259 was consistent with the location of the hydroxyl group at C-9 and cleavage of the bond between C-9 and C-10. The ion at m/z 229 also indicates that the location of the hydroxyl group is C-9, and fragmentation occurs between C-8 and C-9. S-II had UV spectrum identical to authentic 9-HODE with a λ_{max} value of 234–235, which indicated the presence of a conjugated *cis,trans*-hydroxy diene system (Ingram and Brash, 1988). HPLC, GC-MS, and UV analysis indicates that this peak corresponded to 9-hydroxy-10-*trans*,12-*cis*-octadecadienoic acid (9-HODE).

The analysis of the chirality of HODEs showed that 9(R)-HODE and 13(S)-HODE were the main enantiomers formed by dermal fibroblasts **(Fig 3)**. The ratio between the enantiomers (S:R) formed by fibroblasts were 3.55 ± 0.25 and 4.81 ± 0.81 for 13-HODE; and 0.26 ± 0.04 and 0.22 ± 0.02 (mean ± SD) for 9-HODE, in untreated and IL-1β-treated cells, respectively.

PGHS Activity and PGHS-2 Expression Was Induced by IL-1β To observe the effect of IL-1β on transcription of PGHS-1 and PGHS-2 genes, cells were incubated in the presence of 10 U IL-1β per ml for several periods of time. Expression of specific mRNA encoding for PGHS-1 and -2 was induced by IL-1β in a time-dependent manner **(Fig 4)**. Transcription of PGHS-1 was slightly induced (2.2-fold), whereas PGHS-2 was dramatically induced by IL-1β (31-fold). The maximum levels of PGHS mRNA were observed between 6–9 h of exposure to IL-1β at 10 U per ml.

The western blot analysis of the COX isoforms from membranes of HUV-EC-C cells is shown in **Fig 5**. We measured the time course of PGHS-1 and PGHS-2 protein expression by incubating fibroblasts with 10 U IL-1β per ml for various time intervals. No cross-reactivity was observed between PGHS-1 antiserum and PGHS-2 protein isolated from sheep placenta at the concentrations used in this assay, and PGHS-2 antiserum did not recognize PGHS-1 protein isolated from ram seminal vesicles. The antibody against PGHS-1 recognized a double band corresponding to the migration of purified PGHS-1. Apparently, no variation of PGHS-1 protein was produced as a function of time of treatment with IL-1β. In contrast, after IL-1β treatment there was a significant increase of PGHS-2 protein expression in a time-dependent manner with a maximum between 9 and 24 h of exposure to IL-1β, followed by a decrease at 48 h. The antibody against PGHS-2 peptide recognized a major band corresponding to the migration of purified PGHS-2.

In parallel experiments, PGHS activity as a function of duration of IL-1β-treatment was evaluated as the production of PGs after incubation of cells with 25 μM [^{14}C]AA. The PGs derived from [^{14}C]AA formed by fibroblasts were PGE_2 and PGI_2 (determined as 6-keto-$PGF_{1\alpha}$) as the major compounds, and also minor amounts of

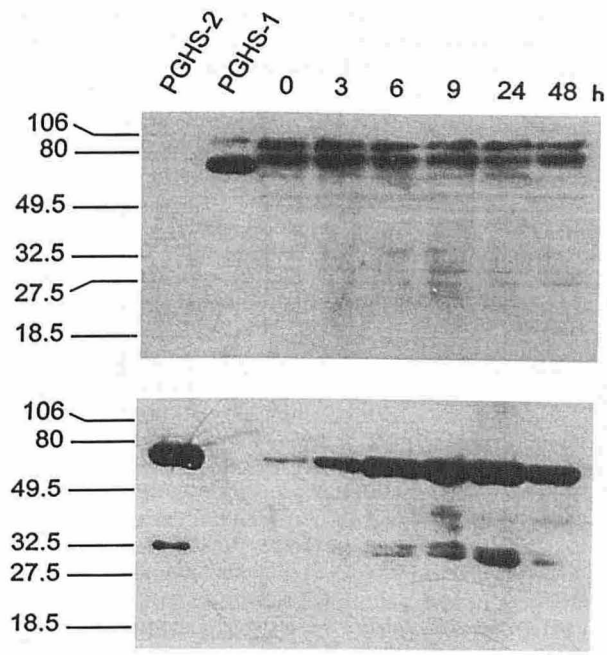

Figure 5. IL-1β caused a time-dependent PGHS-2 expression in dermal fibroblasts. *Upper panel*, representative western blot of PGHS-1 of samples (65 μg of protein) from cells incubated with 10 U IL-1β per ml for the indicated period of time. *Bottom panel*, representative western blot of PGHS-2 of the same samples as the *upper panel*. Purified PGHS-1 (from ram seminal vesicles, 0.3 μg) and PGHS-2 (from sheep placenta, 0.3 μg) were used as a reference. Location of the standards of molecular mass in kilodaltons is indicated. Similar results were obtained with cell extracts from two different batches of cells.

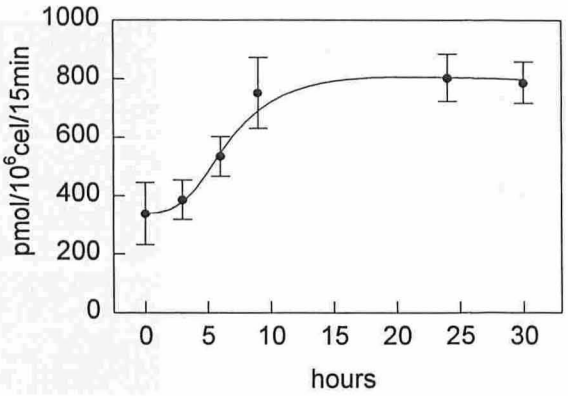

Figure 6. IL-1β caused a time-dependent increase of PGHS activity in dermal fibroblasts. Cells were treated with 10 U IL-1β per ml for the indicated period of time. Afterward, cells were incubated with 25 μM [^{14}C]AA. PGHS activity is expressed as picomoles of [^{14}C]AA transformed into prostanoids (sum of PGE_2, 6-keto-$PGF_{1\alpha}$, and $PGF_{2\alpha}$) by 10^6 cells in 15 min. *Error bars*, mean ± SD; n = 4.

$PGF_{2\alpha}$. PGHS activity estimated as the sum of the aforementioned PGs reached a maximum after 9 h of exposure to the cytokine and was sustained at least for 30 h **(Fig 6)**. Twenty four hours of treatment with IL-1β was chosen to evaluate the effect of IL-1β on LA metabolism in dermal fibroblasts.

IL-1β Increased the Metabolism of LA in Human Dermal Fibroblasts Cells were incubated with different concentrations of IL-1β for 24 h and then incubated with [^{14}C]LA as described in *Materials and Methods*. **Figure 7** depicts the synthesis of HODEs as a function of cytokine concentration, which achieved a maximum at 10 U IL-1β per ml. The EC_{50} values of IL-1β for 13-HODE and 9-HODE formation were 3.2 ± 0.5 and 3.4 ± 0.4 U per ml (mean ± SD), respectively, and quite similar to the EC_{50} value for the sum of the aforementioned PGs (2.6 ± 0.4 U per ml). The time course of the production of LA metabolites in cells treated with

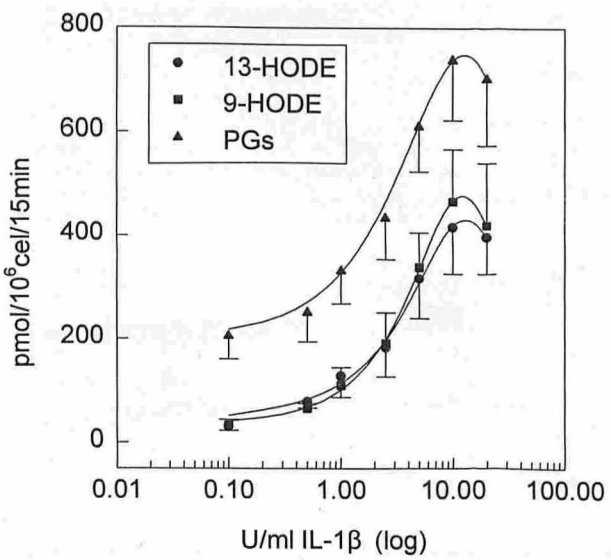

Figure 7. IL-1β caused a parallel concentration-dependent increase of PGs and HODEs biosynthesis by dermal fibroblasts. Cells were treated with IL-1β for several periods of time. Then cells were washed and incubated with 50 μM [^{14}C]LA or 25 μM [^{14}C]AA for 15 min and the products formed were analyzed. The EC_{50} value of IL-1β was 3.2, 3.5, and 2.6 U per ml for 13-HODE, 9-HODE, and PGs, respectively. *Error bars*, mean ± SD; n = 3.

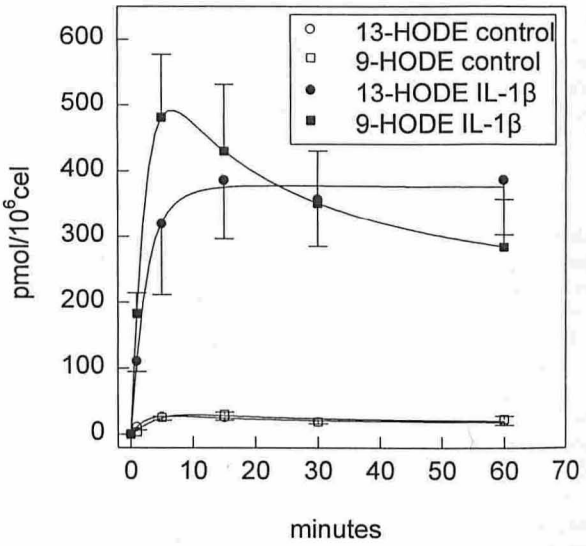

Figure 8. 13-HODE and 9-HODE were formed in the first 5 min of incubation with [^{14}C]LA. Cells were incubated with and without 10 U IL-1β per ml for 24 h and afterward incubated with 50 μM [^{14}C]LA for the indicated periods of time. Points are mean ± SD; n = 3.

IL-1β at 10 U per ml and in untreated cells showed that the levels of HODEs achieved a maximum between 5 and 15 min of incubation, after which a decrease in the levels of 9-HODE was observed **(Fig 8)** during the next 60 min.

Coincubation of cells with IL-1β and 3 μg cycloheximide per ml, 1 μM actinomycin D, or 10 nM dexamethasone abolished the effect of IL-1β on octadecanoid synthesis. No effect of these inhibitors was observed on the production of HODEs by control cells or on cell viability determined by trypan blue dye exclusion.

To demonstrate that the effect of IL-1β was mediated by PGHS, we incubated cells treated with IL-1β for 24 h with increasing concentrations of indomethacin and ASA prior to the incubation with [^{14}C]LA as described in *Materials and Methods*. Results in **Fig 9** showed a concentration-dependent inhibition of the synthesis of both 9-HODE and 13-HODE by the PGHS inhibitors.

In order to determine the cellular location of the enzymatic activity responsible for the biosynthesis of HODEs, we performed experiments with fractionated cells. Microsomal (100,000 × *g* pellet) and cytosolic (100,000 × *g* supernatant) fractions were incubated separately with 50 μM [^{14}C]LA. As shown in **Table I**, the synthesis of both 13-HODE and 9-HODE was mainly associated with the microsomal fraction.

Distribution of Octadecanoids in the Different Classes of Cellular Phospholipids Fibroblasts untreated and treated with IL-1β for 24 h were incubated in the presence of labeled HODEs as described in *Materials and Methods*. Dermal fibroblasts incorporated both 9-HODE and 13-HODE into their phospholipids to a similar extent. The relative degree of incorporation of [^{14}C]13-HODE and [^{14}C]9-HODE into the different classes of phospholipid in both untreated and IL-1β–treated cells was phosphatidylcholine>phosphatidyl-ethanolamine≈sphingomyelin>phosphatidylinositol>phosphatidyl-serine. Results in **Table II** indicate that IL-1β treatment significantly enhanced the ability of 13-HODE to be incorporated into all phosphoglyceride classes analyzed except sphingomyelin. The highest increase in the 13-HODE esterification caused by IL-1β treatment was observed in phosphatidylinositol (2.4-fold). In contrast, no statistically significant effect of IL-1β on 9-HODE incorporation into any phospholipid class was observed.

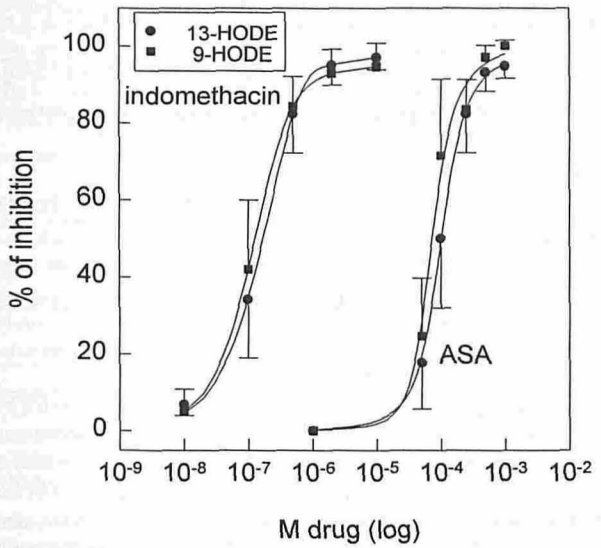

Figure 9. ASA and indomethacin (PGHS inhibitors) inhibited 13-HODE and 9-HODE biosynthesis by IL-1β–treated fibroblasts. Fibroblasts were treated with 10 U IL-1β per ml for 24 h and then incubated with [^{14}C]LA in the presence of the indicated concentration of the drugs. n = 4, mean ± SD. IC_{50} of indomethacin: 0.17 ± 0.04 and 0.13 ± 0.05 μM (mean ± SD) for 13-HODE and 9-HODE, respectively; IC_{50} of ASA: 102 ± 41 and 73 ± 36 μM (mean ± SD) for 13-HODE and 9-HODE, respectively.

DISCUSSION

Our findings demonstrate that human dermal fibroblasts produce 13-HODE and 9-HODE as the main metabolites of LA. IL-1β stimulates the synthesis of both compounds in a time and concentration-dependent manner. 13-HODE may be synthesized through both the PGHS and the 15-LO pathways, depending on the cell type or tissue, whereas 9-HODE is produced enzymatically only by PGHS (Hamberg and Samuelsson 1967; Reinaud *et al*, 1989; Camacho *et al*, 1995). Despite the fact that 15-LO activity has been inferred to be present in dermal fibroblasts (Mayer *et al*, 1984; Kragballe *et al*. 1986), experimental evidence supports the conclusion that PGHS-2 was the main enzyme involved in the increase in the formation of both 13-HODE and 9-HODE by IL-1β: (i) The PGHS inhibitors, ASA and indomethacin, inhibited the synthesis of both 13-HODE and 9-HODE in IL-1β–treated cells, and the recovery of 13-HODE and 9-HODE synthesis after ASA treatment (irreversible inhibitor of PGHS) showed a parallel recovery of 13-HODE and 9-HODE (data not shown), which suggests a common enzymatic pathway; (ii) the EC_{50} values of IL-1β for both 13-HODE and 9-HODE were almost identical, which also indicates a common enzymatic pathway; (iii) the ratio of enantiomers of HODEs in controls and in IL-1β were quite similar to those obtained when HODEs are synthesized through isolated PGHS-1 and PGHS-2, whereas isolated 15-LO yields strict S configuration

Table I. 13-HODE and 9-HODE Were Mainly Produced by the Microsomal Fraction[a]

		13-HODE	9-HODE
100,000 × *g* supernatant	Controls	3.40 ± 4.8	7.16 ± 10.1
	IL-1β	4.56 ± 9.5	5.50 ± 4.9
100,000 × *g* Pellet	Controls	55.4 ± 17.0	72.27 ± 13.2
	IL-1β	174.11 ± 19.2	253.03 ± 15.5

[a] Fibroblasts fractions were incubated with 50 μM [^{14}C]LA at 37°C for 10 min. Octadecanoids were extracted and analyzed. Results are expressed as picomoles of each compound produced by milligrams of protein in 10 min. Mean ± SD, n = 4.

Table II. HODEs Were Mainly Associated to PhC and IL-1β Increase the Esterification of 13-HODE Mainly into PhI[a]

	13-HODE		9-HODE	
	Controls	IL-1β	Controls	IL-1β
PhI	2.78 ± 0.37	6.63 ± 0.95[b]	6.46 ± 1.07	5.98 ± 0.76
PhS	2.58 ± 0.21	4.35 ± 0.28[b]	2.77 ± 0.47	3.96 ± 0.98
PhC	108.3 ± 17.2	165.9 ± 15.59[b]	98.3 ± 3.75	117.8 ± 9.1
PhE	25.7 ± 3.96	40.9 ± 6.34[b]	34.4 ± 5.33	29.5 ± 6.15
SPh	28.0 ± 4.49	25.8 ± 5.19	21.8 ± 1.51	16.8 ± 2.13

[a] Fibroblasts untreated and treated with IL-1β for 24 h were incubated with labeled HODEs for 3 h, and the radioactivity associated to the indicated phospholipid classes was evaluated. Results are expressed as picomoles of [^{14}C]HODEs incorporated in 3 h. Mean ± SD, n = 4.

[b] Significantly higher when compared with controls ($p < 0.05$). PhI, phosphatidylinositol; PhS, phosphatidylserine; PhC, phosphatidylcholine; PhE, phosphatidylethanolamine; SPh, sphingomyelin.

for 13-HODE (Camacho *et al*, 1995); (iv) The subcellular localization of the HODEs' biosynthetic activity was consistent with a PGHS activity rather than 15-LO, because the latter is a cytosolic enzyme (Sigal *et al*, 1988) whereas PGHS is membrane-associated (Morita *et al*, 1995); and finally, (v) the effect of IL-1β on HODEs formation was well correlated with the *de novo* expression of PGHS-2 and with the increase in the PGHS-activity.

The presence of dexamethasone abolished the effect of IL-1β on the production of 13-HODE and 9-HODE. It has been suggested that dexamethasone may act on PGs' release by promoting the synthesis of a protein that inhibited the synthesis of the new PGHS induced by IL-1 (Raz *et al*, 1989). Our results are consistent with this explanation since dexamethasone abolished the production of HODEs only in IL-1 treated cells without affecting the production in nonstimulated cells (data not shown).

HODEs, mainly 13-HODE, are present in high amounts in psoriatic lesions (Camp *et al*, 1983; Baer *et al*, 1990, 1991). Baer *et al* (1991) had pointed out that the chiral analysis of these compounds agrees with a PGHS rather than with a 15-LO origin. Our results demonstrate that this occurs in dermal fibroblasts and that this cell type may contribute to the HODEs detected in psoriatic lesions. The effect of IL-1β on the stimulation of LA metabolism could also explain the high levels of these compounds found in the injured skin and suggests that all factors that increase the expression of PGHS-2 may act on fibroblasts by increasing the synthesis of HODEs. Another point that supports the role of PGHS-2 in the biosynthesis of HPODEs and HODEs is that LA is a substantially better substrate for PGHS-2 than for PGHS-1 (Camacho *et al*, 1995).

The role of LA-derived octadecanoids in inflammatory and proliferative diseases is not clear. Nevertheless, a growing amount of data suggest that octadecanoids derived from LA play a role as elements of signal pathways necessary for cell mitogenesis when stimulated by growth factors or oncogenic transformation. In particular, LA, HPODEs, and HODEs enhance the response of epithelial cells and fibroblasts to insulin and epidermal growth factor, respectively (Bandyopadhyay *et al*, 1987; Glasgow *et al*, 1992). The proliferative effect of free hydroperoxides derived from LA is mediated by activation of a protein kinase in response to mitogens and c-*fos*, c-*jun*, and c-*myc* mRNA expression (Rao *et al*, 1995).

In contrast, Miller *et al* (1990) reported that 13-HODE reverses the epidermal hyperproliferation induced in essential fatty acids–deficient conditions. Nevertheless, this activity seems to be related to the esterification of 13-HODE in the phospholipids mainly into phosphatidylcholine and phosphatidylinositol and the generation of 13-HODE–containing diacylglycerol (Cho and Ziboh, 1994a), rather than with an activity of free 13-HODE by itself. This oxidized diacylglycerol inhibits epidermal protein kinase C isoenzymes, which are involved in the regulation of cellular proliferation

(Cho and Ziboh, 1994b), and its activity is elevated in psoriasis, consistent with the high levels of diacylglycerol (Fisher *et al*, 1990).

The precise role of oxidized phospholipids is uncertain, but 13-HODE was found to be the main hydroxy-fatty acid esterified in psoriatic scales (Baer *et al*, 1990, 1991). The fact that IL-1β may also increase the esterification of 13-HODE may contribute to the high levels of 13-HODE associated with phospholipids found in skin lesions.

The fact that oxidized LA modulates signal transduction of the mitogenic response and that elevated levels of free and esterified HODEs have been found in samples from inflammatory proliferative diseases such as atherosclerosis (Künn *et al*, 1992) and psoriasis (Camp *et al*, 1983; Baer *et al*, 1990, 1991) suggests that these compounds play a role in the physiopathology of such diseases. Although epidermal cells may synthesize 13-HODE (Nugteren and Kivits, 1987), there is no information, as far as we know, concerning the contribution of dermal fibroblasts to the HODEs pool found in the inflammatory lesions on skin.

In vivo, the limiting step for octadecanoid formation may be the amount of free LA. The high levels of these compounds found in psoriatic lesions could be, in part, a consequence of the stimulation of their biosynthesis by cytokines such as IL-1 through the PGHS-2 expression on skin cells including endothelial (Camacho *et al*, 1995) and epidermal cells (Scholz *et al*, 1995) or/and of the release of those previously esterified in membrane lipids. Another source of 13-HODE could be the 15-LO present in infiltrating leukocytes (Sigal *et al*, 1988) and epidermal cells (Nugteren and Kivits, 1987). More studies are needed to assess the significance of these results *in vivo*, and these should include studies of the effect of potent and selective PGHS-2 selective inhibitors, which are at present being developed (Aslanian *et al*, 1994), on skin inflammatory diseases.

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