Pergamon

0006-2952(95)00211-1

Biochemical Pharmacology, Vol. 50, No. 7, pp. 913–922, 1995.

0006-2952/95 $9.50 + 0.00

REGULATION OF PROSTAGLANDIN H SYNTHASE 2 EXPRESSION IN HUMAN MONOCYTES BY THE MARINE NATURAL PRODUCTS MANOALIDE AND SCALARADIAL

NOVEL EFFECTS INDEPENDENT OF INHIBITION OF LIPID MEDIATOR PRODUCTION

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(*Received* 22 *April* 1994; *accepted* 23 *March* 1995)

Abstract—The marine natural products manoalide and scalaradial are potent anti-inflammatory agents that inactivate the enzyme phospholipase A_2 (PLA_2) *in vitro*. To study the mechanism of inhibition of prostaglandin E_2 (PGE_2) production in human monocytes by manoalide and scalaradial, lipopolysaccharide (LPS)-induced prostaglandin biosynthesis and induction of prostaglandin H synthase (PGHS) were evaluated. LPS (10 ng/mL) and interleukin-1β (IL-1β, 50–1000 ng/mL) but not tumor necrosis factor α (TNFα, 300 ng/mL) induced the expression of the PGHS-2 isoform as determined by immunoblot analysis with a specific polyclonal antibody for PGHS-2. Manoalide and scalaradial (1–10 μM) inhibited LPS-induced endogeneous PGE_2 production, reduced the LPS-induced PGHS activity, and reduced the expression of PGHS-2. Indomethacin [a PGHS inhibitor (0.01 to 0.1 μM)], zileuton [a 5-lipoxygenase inhibitor (3–10 μM)], and WEB-2806 [a platelet-activating factor (PAF) antagonist (30 μM)] did not affect the LPS-induced expression of PGHS-2 in human monocytes. These results suggest that modulation of lipid mediator production by manoalide or scalaradial may not be involved in the observed effects on the expression of PGHS-2. Manoalide and scalaradial also inhibited the release of IL-1β and TNFα from LPS-stimulated monocytes. Expression of PGHS-2 induced by either LPS or IL-1β was blocked by the IL-1 receptor antagonist (IL-1ra, 2 μg/mL) but not by rolipram, a phosphodiesterase IV inhibitor that inhibits TNFα but not IL-1β release. Similar to LPS, IL-1β-induced PGHS-2 expression was apparently not regulated by lipid mediators such as prostaglandins, leukotrienes or PAF as determined with specific inhibitors and antagonists. Scalaradial and to some extent manoalide were capable of blocking the IL-1β-induced expression of PGHS-2. These results indicate that IL-1β is the predominant cytokine responsible for the induction of PGHS-2 in the human monocyte. Furthermore, marine natural products such as scalaradial have novel effects on the IL-1β-mediated induction of PGHS-2 in human monocytes, which appears to be independent of effects on lipid mediator production.

Key words: cyclooxygenase (prostaglandin G/H synthase); scalaradial; manoalide; interleukin-1β; interleukin-1 receptor antagonist; human monocyte; rolipram; WEB-2086; SKF-86002; LPS (lipopolysaccharide)

The metabolism of arachidonic acid into the pro-inflammatory prostaglandins is a crucial event in the development of inflammation. It has long been recognized that the enzyme cyclooxygenase (PGHS†, EC 1.14.99.1) is the key enzyme in the production of prostaglandins and is the target of the aspirin-like NSAIDs. The capacity for cells to produce prostaglandins requires the regulation of two different enzymes: PLA_2 which liberates arachidonic acid from phospholipid stores, and PGHS, which oxygenates the arachidonic acid to form the prostaglandins. Elevated prostaglandin levels at inflammatory sites may arise from the up-regulation or induction of PLA_2 [1] and/or PGHS [2], both of which have been observed in several cell types in response to various inflammatory stimuli [3–5].

In fibroblasts, the up-regulation of PGHS occurs in the presence of IL-1β [2, 6], and in human monocytes [7] and rabbit alveolar macrophages [8, 9] the up-regulation occurs with both LPS and IL-1β. The induction of PGHS in the presence of IL-1β was blocked by protein synthesis inhibitors (cyclohexamide) and by dexamethasone [6]. The dexamethasone response was also inhibited by the transcription inhibitor actinomycin D and suggested a very interesting cascade of proteins involved in lipid mediator regulation by glucocorticoids with the terminal regulation of an inducible form of PGHS [6]. Until recently, this was attributed to the up-regulation of a single form of PGHS; however, a second gene for PGHS was found and shown to be regulated by mitogenic stimulation [10, 11].

The original gene isolated for PGHS was encoded by a 2.8-kb mRNA as determined from sheep seminal vesicles [12], a rich source of PGHS, and from human endothelial cells [13–15]. When cells were stimulated with either serum or inflammatory agents and the northern analyses done under low stringency conditions, a second much larger mRNA species (4.0 to 5.0 kb) was detected [16]. This form of PGHS was encoded by a 4.5-kb mRNA and was found only when induced [10, 11, 17]. PGHS-2 (also known as COX-2, mi-COX and prostaglandin endoperoxide synthase 2) is very homologous with the constitutive 2.8-kb form designated PGHS-1 (also known as COX, COX-1 or prostaglandin endoper-

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† Abbreviations: PGHS, prostaglandin H synthase; PGHS-2 or mi-COX, prostaglandin H synthase 2; IL-1β, interleukin-1β; TNFα, tumor necrosis factor α; PGE_2, prostaglandin E_2; LTB_4, leukotriene B_4; LPS, lipopolysaccharide; PLA_2, phospholipase A_2; PMA, phorbol myristate acetate; IL-1ra, interleukin-1 receptor antagonist; PAF, platelet-activating factor; and NSAIDs, nonsteroidal anti-inflammatory drugs.

oxide synthase 1). At the protein level these two isoforms display 60% identity and conservation of key catalytic and aspirin binding residues as well as numerous glycosylation sites [17]. An interesting feature of this PGHS-2 is that it is found only in the presence of an inflammatory stimulus (e.g. LPS or IL-1β) and, therefore, may be an important isozyme involved in the regulation of the inflammatory response [5]. Selective inhibition of the inflammatory cyclooxygenase, PGHS-2, may circumvent some of the known liabilities of inhibition of COX-1 by currently available NSAIDs [18, 19].

We have reported previously that manoalide and scalaradial have differential effects on PGE_2 production in human monocytes, depending on the stimulus utilized [20]. Herein we report on the regulation of eicosanoid biosynthesis and the induction of PGHS-2 in purified human peripheral monocytes stimulated with LPS and IL-1β by various inhibitors of lipid mediator production including the marine natural products manoalide and scalaradial.

MATERIALS AND METHODS

Materials

LPS (*Escherichia coli* 0111:B4) was purchased from List Biologicals (Campbell, CA). Polyclonal anti-PGHS-2 was purchased from the Cayman Chemical Co. (Ann Arbor, MI). Purified ram seminal vesicle PGHS-1 and recombinant PGHS-2 were purchased from Oxford Biochemicals (Oxford, U.K.). Manoalide and 12-*epi*-scalaradial were purchased from Biomol (Philadelphia, PA). IL-1β and TNFα were purchased from Genzyme (Cambridge, MA). IL-1ra was purchased from R&D Systems (Minneapolis, MN). Zileuton (A64077) was provided by Abbott Laboratories. Rolipram was synthesized by Wyeth-Ayerst Research. WEB-2086 was provided by Boehringer Ingelheim. Indomethacin, PMA, and ionomycin were purchased from the Sigma Chemical Co. (St. Louis, MO). EIA kits for PGE_2 and LTB_4 were obtained from the Cayman Chemical Co. IL-1β and TNFα ELISA kits were purchased from Cistron (Pinebrook, NJ). ECL reagents and film were purchased from Amersham (Rockford, IL). Immobilon-P membranes were purchased from Millipore (Bedford, MA).

Isolation of human monocytes

Leukocyte-enriched blood samples obtained from healthy male donors were procured by leukophoresis using a Haemonetics model V-50+ blood processor (Biological Specialties, Inc., Lansdale, PA). The top "platelet-rich" layer was removed after a low-speed spin (35 *g*, 10 min, 25°). The remaining cell suspension was centrifuged (400 *g*, 10 min, 25°), then washed once with HBSS (Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}), and resuspended into 120 mL HBSS. To aliquots of 30 mL each, 10 mL of Histopaque 1077 (Sigma) was carefully layered at the bottom of each tube, and the cells were separated by centrifugation (400 *g*, 30 min, 25°). The monocyte-rich layers (buffy layer) were collected, washed once by centrifugation using HBSS, and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) with 5% fetal bovine serum (FBS) or 1% heat-inactivated autologous serum (HIAS) and 2 mM L-glutamine.

Cell sorting by Biomag antibodies using negative selection

The T and B lymphocytes were removed by negative selection using the magnetic anti-CD 3 and magnetic anti-CD 19 antibodies, respectively (Advanced Magnetic Inc., Boston, MA). Before use, the magnetic antibodies were washed twice with HBSS. An aliquot of approximately 1×10^9 cells in IMDM with 5% FBS or 1% HIAS was mixed with magnetic anti-CD 3 antibody (10 mL) and anti-CD 19 antibody (10 mL). The mixture was rocked for 1 hr at 4° and then placed on a magnetic separation unit for 10 min. The supernatant was aspirated and diluted to 1×10^7 cells/mL in IMDM. The monocytes (1×10^7/mL) were further purified by adherence in a 6-well tissue culture plate for 1 hr at 37°. After adherence, the wells were washed three times with IMDM to remove any contaminating lymphocytes.

Eicosanoids and eicosanoid EIA

Purified monocytes were preincubated with compound in DMSO (final DMSO concentration was 0.1%) or DMSO vehicle for 30 min at 37°. For the ionomycin (2 μM)/PMA (20 nM) studies, after preincubation the cells were stimulated for 30 min at 37°. For LPS induction studies, after preincubation the cells were stimulated with LPS [10 ng/mL *E. coli* 0111:B4 (List)] for 18 hr at 37°. An aliquot of 50 μL/well was taken for endogenous PGE_2 production. Total cellular cyclooxygenase activity was determined by removing the LPS-containing medium from the cells (after 18 hr) and replacing it with medium that contained arachidonic acid (30 μM) and incubating for 10 min at 37°. PGE_2 contained in the medium after arachidonic acid was taken as total cellular cyclooxygenase activity. PGE_2 and LTB_4 were assayed by EIA (Cayman Chemical Co.).

Electrophoresis and immunoblotting

After LPS stimulation, monocyte monolayers were lysed with Triton X-100 lysis buffer [50 mM Tris–HCl (pH 8.0), 10% glycerol, 137 mM NaCl, 1.5% Triton X-100] for 1 hr at 4° with rocking. SDS–PAGE sample buffer (2×) was added, and the well was scraped to remove any residual cells. The samples were boiled at 100° for 10 min and centrifuged to pellet debris; the supernatant was frozen at −70° until SDS–PAGE analysis. SDS–PAGE was performed using 10% PAGE mini-gels (Diiachi). Samples (20 μL) were loaded and electrophoresed at 50 mA/gel for 1.0 hr. After pre-equilibration of gels in transfer buffer (192 mM glycine, 25 mM Tris, 15% methanol, pH 8.3) for 30 min, the proteins were transferred to Immobilon-P (Millipore) membranes using a BioRad mini-gel transfer unit (100 V for 1.5 hr). After transfer, the membranes were washed with PBS and then blocked with 3% non-fat milk in PBS (pH 7.4) for 1 hr at 22°. Primary antibody (PGHS-2 specific rabbit polyclonal IgG, Cayman Chemical Co.) at 10 μg/mL in 3% non-fat milk in PBS (pH 7.4) was incubated with the membrane overnight at 4°. The membranes were washed several times with PBS and then incubated with secondary antibody [goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate, Boehringer Mannheim] at a 1:10,000 dilution in 3% non-fat milk in PBS for 1 hr at 22° with rocking. After the secondary antibody, the membranes were washed extensively with 0.05% Tween-20 PBS (10 × 5 min). Proteins of interest were

then visualized using the enhanced chemiluminescence method as described by the manufacturer (Amersham). The PGHS-2 protein purchased as an SDS–PAGE standard was from Oxford Biochemicals and is an *E. coli* expressed unglycosylated protein that has a faster mobility on SDS–PAGE than native monocyte PGHS-2. Multiple bands associated with the PGHS-2 immunoblots represent different glycosylated species of the enzyme. PGHS-2 Ab (Cayman Chemical Co.) demonstrated no cross-reactivity with up to 10 μg/lane of ovine PGHS-1. Residual bands at 30 min in resting monocytes may be associated with leukophoresis methods used to obtain the cells.

RESULTS

Effects of manoalide, scalaradial and dexamethasone on monocyte eicosanoid production

Human monocytes were stimulated with a combination of ionomycin (2 μM) and PMA (20 nM) for 30 min at 37° after preincubation for either 30 min or 18 hr with inhibitors. The major eicosanoids produced by these monocytes were PGE_2 and LTB_4. As shown in Fig. 1 (top panel), manoalide, scalaradial and dexamethasone did not inhibit PGE_2 production when monocytes were preincubated with compounds for only 30 min, whereas an 18-hr preincubation produced a concentration-dependent inhibition of PGE_2 production for manoalide and scalaradial. Dexamethasone produced only a slight inhibition (20%) of PGE_2 production when preincubated with monocytes for 18 hr (Fig. 1, top panel). LTB_4 production was inhibited by manoalide and scalaradial regardless of the preincubation time employed (Fig. 1, bottom panel). These results suggested that manoalide and scalaradial may have selective effects on an induction process involved in the regulation of PGE_2 production (e.g. induction of cyclooxygenase).

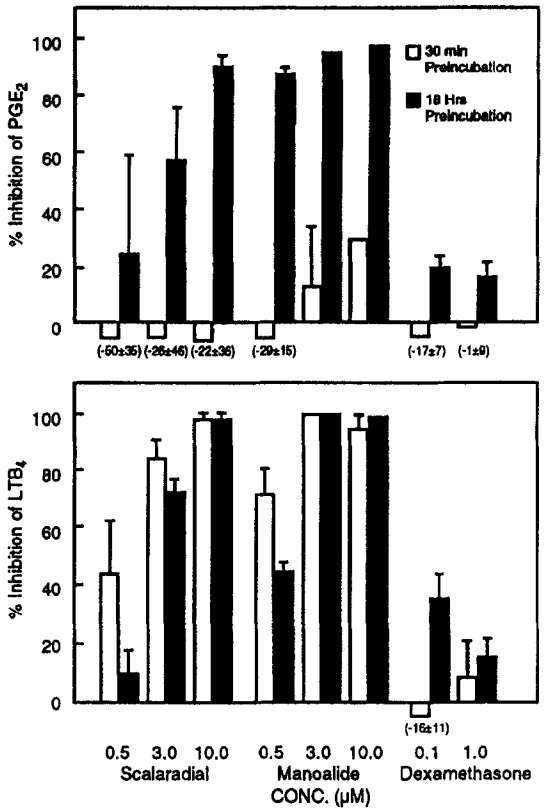


Fig. 1. Effect of preincubation time with inhibitors on ionomycin/PMA-stimulated eicosanoid production. Human monocytes were preincubated with inhibitors for either 30 min (□) without serum or 18 hr (■) with 5% FBS. PGE_2 and LTB_4 were measured after 30-min stimulation with ionomycin (2 μM)/PMA (20 nM) in the absence of serum by EIA. Cell viability was >90% in all groups tested. Percent inhibition values are means ± SEM from three separate donors (N = 3). Total PGE_2 production ranged from 0.63 to 1.68 ng/5 × 10^6 cells for the 30-min preincubation samples and from 0.55 to 7.76 ng/5 × 10^6 cells for the 18-hr preincubation samples. Total LTB_4 production ranged from 2.73 to 7.64 ng/5 × 10^6 cells for the 30-min preincubation samples and from 1.76 to 6.45 ng/5 × 10^6 cells for the 18-hr preincubation samples.

Induction of PGHS-2, IL-1β, and TNFα by LPS and effects of inhibitors

To evaluate the effects of various inhibitors on monocyte PGE_2 production, a known inducer of cyclooxygenase was used, bacterial LPS [8, 21]. When monocytes were exposed to LPS (10 ng/mL) for 30 min, the level of PGHS activity (measured as total cellular PGHS activity by the exogenous application of 30 μM arachidonic acid) did not change nor did a protein synthesis inhibitor (cyclohexamide, 10 μM) have any effect on this PGHS activity. However, after 18 hr of LPS treatment, PGHS activity was increased by approximately 20-fold (data not shown). This induction of PGHS activity was blocked by cyclohexamide (10 μM) and returned to basal levels, suggesting that only the induced PGHS was affected by the cyclohexamide treatment (as shown by others) [2, 6].

To study the effects of LPS on the induction of PGHS activity, specific antibodies to PGHS-1 and PGHS-2 were used to evaluate changes in PGHS isozyme protein levels in cells by western analysis. The time courses of PGE_2 production and PGHS-2 expression are shown in Fig. 2. PGE_2 production in LPS-treated cells increased steadily between 6 and 18 hr. This major increase in PGE_2 production lagged somewhat behind the majority of the production of IL-1β and TNFα, which reached near maximum levels by 6 hr of LPS treatment (Fig. 2). The increase in PGE_2 levels appeared to correlate with an increase in immunoreactive PGHS-2, as determined by a PGHS-2 specific polyclonal antibody. No change in PGHS-1 levels was noted using specific antibodies, nor was there any cross-reactivity of the PGHS-2 antibody with PGHS-1 (data not shown). Similar amounts of immunoreactive PGHS-2 were found using 1 μg/mL LPS. Therefore, a more physiologically relevant LPS concentration of 10 ng/mL was used in all subsequent experiments.

The effects of various inhibitors on the endogenous release of PGE_2 induced by LPS and on the total cellular PGHS activity were then compared (Fig. 3). LPS-stimulated PGE_2 production was concentration-dependently inhibited by manoalide, scalaradial, dexamethasone and indomethacin (Fig. 3, upper panel). Interestingly, dexamethasone was able to inhibit LPS-stimulated PGE_2 production, whereas it did not inhibit the acute (30 min) ionomycin/PMA-stimulated PGE_2 production. Cyclohexamide also inhibited the LPS-induced PGE_2 production (Fig. 3, upper panel). The effects observed on endogenous PGE_2 production could not be explained to-

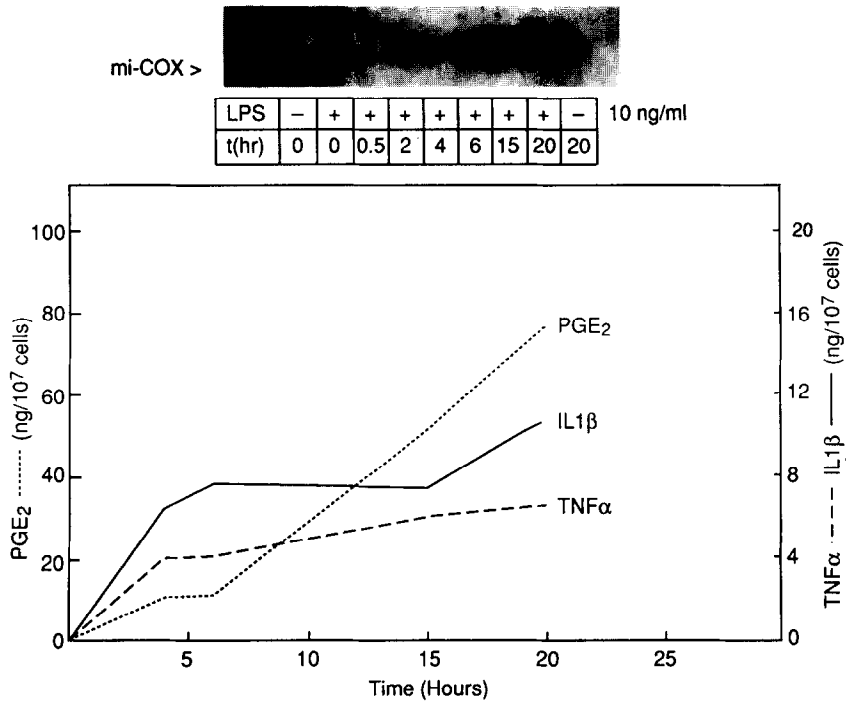


Fig. 2. Time courses for the release of PGE_2, IL-1β, TNFα and the expression of PGHS-2 in LPS-treated monocytes. PGE_2, IL-1β, and TNFα were measured in the culture medium at 4, 6, 15 and 20 hr of LPS treatment. PGHS-2 (mi-COX) immunoblot samples were taken at 0.5, 2, 4, 6, 15, and 20 hr and processed as described in Materials and Methods. Data are representative of at least two experiments (donors) showing similar results.

tally by the prevention of PGHS expression, inasmuch as manoalide and scalaradial are potent inhibitors of PLA_2. Therefore, these drugs would be expected to inhibit PGE_2 production by blocking arachidonic acid release. To determine the effects of these inhibitors on the expression of PGHS, total cellular PGHS activity was evaluated using exogenously applied arachidonic acid. As shown in Fig. 3 (lower panel), unexpectedly, manoalide and scalaradial concentration-dependently inhibited the LPS-induced PGHS activity in monocytes. Manoalide and scalaradial have been shown to have no direct effects on PGHS enzymes when evaluated in cellular systems (e.g. human polymorphonuclear leukocytes or murine macrophages determined by metabolism of exogenous arachidonic acid); however, this does not exclude a direct effect of manoalide and scalaradial on human PGHS-1 and PGHS-2. Preliminary data demonstrated inhibition of human recombinant PGHS-1 and PGHS-2 in the 1–10 μM range in an *in vitro* enzyme assay (Glaser KB, unpublished observations). Therefore, the inhibition of PGHS activity in Fig. 3 (lower panel) may reflect both inhibition of expression and activity, which results in nearly complete loss of activity. The inhibitors evaluated (with the exception of indomethacin, a direct PGHS inhibitor) did not inhibit PGHS activity below the background or unstimulated levels, indicating that their effect was only on that induced by LPS (Fig. 3, lower panel).

Manoalide and scalaradial inhibit both PGE_2 production and PGHS-2 expression in human monocytes. Since LPS also induces the production of IL-1β and TNFα, their effects on cytokine production were investigated. As shown in Fig. 4, manoalide and scalaradial (1–10 μM) and dexamethasone (0.1 to 1.0 μM) inhibited the production of IL-1β (upper panel) and TNFα (lower panel) in response to LPS. To test whether the reduction in IL-1β and TNFα production was due to the inhibition of PGE_2 production and its subsequent autocoid functions, indomethacin was evaluated at concentrations that completely prevent PGE_2 production (Fig. 4). Indomethacin at 0.01 and 0.1 μM had no effect on IL-1β or TNFα production (Fig. 4, upper and lower panels, respectively).

Inhibitor effects on PGHS-2 induction

Since manoalide, scalaradial and dexamethasone inhibit the induction of PGHS activity in response to LPS, their effect on PGHS-2 expression, as determined by western analysis, was evaluated. In these monocyte preparations, a basal level of PGHS-2 was always detected at time 0 or after 30 min of LPS treatment (Fig. 5). After 30 min of LPS treatment, there was no detectable change in the amount of immunoreactive PGHS-2 in the monocytes. After 18 hr of LPS treatment, there was a marked induction of the immunoreactive PGHS-2, which was inhibited by treatment of monocytes with cyclohexamide (Fig. 5). Dexamethasone at 0.1 μM abolished the induction of PGHS-2 by LPS, as previously shown by others [22–27]. Inhibition of PGHS-2 induction by cyclohexamide or dexamethasone did not alter the basal levels of either PGHS activity or the immunoreactive protein in these studies. Manoalide (1–10 μM) and scalaradial (1–10 μM) concentration-dependently inhibited the induction of PGHS-2 by LPS (Fig. 5). The inhibition of immunoreactive PGHS-2 appeared to correlate with the reduction in PGHS activity in monocytes

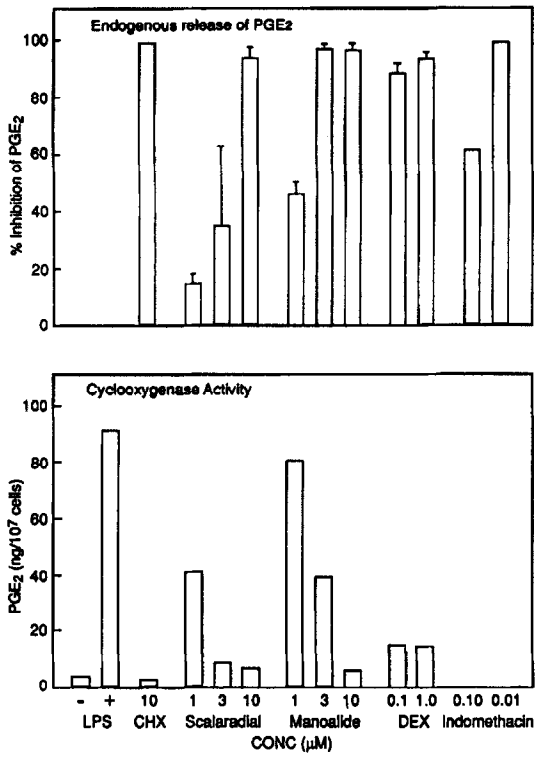


Fig. 3. Effects of various inhibitors on LPS-treated monocyte PGE_2 production and PGHS activity after 18 hr of preincubation. Monocytes were treated with LPS (10 ng/mL) for 18 hr with 5% FBS plus or minus various inhibitors. Endogenous release of PGE_2 (upper panel) was determined from culture medium after 18 hr of LPS treatment (mean ± SEM, N = 3 donors; total PGE_2 production ranged from 10.75 to 30.20 ng/10^7 cells). Cyclooxygenase (PGHS) activity (lower panel) was determined by addition of exogenous arachidonic acid (30 μM) and incubation for 10 min and subsequently measuring PGE_2 by EIA. Abbreviations: CHX, cyclohexamide; and DEX, dexamethasone. These data are taken from one donor and are representative of at least two other donors showing similar results.

treated with these compounds. They did not reduce either PGHS activity or PGHS-2 immunoreactivity below the basal levels present in these monocytes.

As shown for IL-1β and TNFα production, indomethacin (0.1 μM) did not inhibit the induction of PGHS-2 in monocytes when complete inhibition of PGHS activity occurred (data not shown).

Evaluation of other mediator inhibitors on the induction of PGHS-2

To evaluate the mechanism of inhibition of PGHS-2 expression by manoalide and scalaradial, various other inhibitors of signal transduction and lipid mediator production were investigated. As demonstrated in the previous sections, the cyclooxygenase inhibitor indomethacin did not affect the LPS-stimulated induction of IL-1β, TNFα or PGHS-2. Other lipid mediator inhibitors, such as the PAF antagonist, WEB-2086, and the 5-lipoxygenase inhibitor zileuton (A64077), also had no effect on the LPS-stimulated induction of PGHS-2 (Fig. 6).

The effect of SKF-86002, a 5-lipoxygenase/cyclooxygenase inhibitor and cytokine release inhibitor [28], on LPS-induced PGHS-2 expression is shown in Fig. 6 (upper panel). SKF-86002 at 3 and 10 μM significantly inhibited the expression of PGHS-2. The role of reactive oxygen species (ROS) in the regulation of transcriptional events [29] was evaluated using nordihydroguaiaretic acid (NDGA). NDGA at 3 and 10 μM inhibited the expression of PGHS-2 in LPS-induced monocytes (Fig. 6, lower panel).

To understand the role of IL-1β and TNFα in the induction of PGHS-2, the phosphodiesterase IV (PDE-IV) inhibitor rolipram was used to inhibit TNFα production, and IL-1ra was used to inhibit IL-1β effects. Rolipram at concentrations that reduce TNFα production by >95% had no effect on the induction of PGHS-2 by LPS (Fig. 6, lower panel). Likewise, rolipram did not inhibit the production of PGE_2 in LPS-stimulated monocytes (data not shown), indicating that prostaglandin production in LPS-stimulated human monocytes is probably not mediated by PDE-IV-regulated cAMP; however, it is apparent that this cAMP does regulate TNFα production. To rule out any direct effect of TNFα on PGHS-2 induction, we determined that TNFα at concentrations of 250 ng/mL (18 hr) failed to induce PGHS-2 expression in human monocytes (data not shown). Regulation of IL-1β was achieved by use of IL-1ra to prevent IL-1β-induced effects on the monocyte. A concentration-dependent inhibition of LPS-induced PGHS-2 expression was observed with IL-1ra between 0.1 and 1.0 μg/mL (data not shown). Since the level of IL-1β generated by LPS in these monocytes is approximately 10 ng/mL, the concentration of IL-1ra employed was a 10- to 100-fold excess, which is sufficient to block >85% of all IL-1-mediated effects [30, 31]. This concentration was sufficient to inhibit PGHS-2 expression (Fig. 6, upper panel). As shown in Fig. 7, IL-1ra concentration-dependently blocked the induction of PGHS-2 with an apparent IC_{50} of 0.8 μg/mL in response to 10 ng/mL of LPS. IL-1ra also concentration-dependently blocked IL-1β-induced PGHS-2 expression as expected (Fig. 7).

Induction of PGHS-2, PGE_2 and TNFα production by IL-1β and effects of inhibitors

The effects of different classes of inhibitors on IL-1β-induced PGE_2 and TNFα release as well as PGHS-2 expression in human monocytes were examined. IL-1β from 50 to 1000 ng/mL induced the production of PGHS-2 (Fig. 8, upper panel), and of PGE_2 and TNFα (data not shown) in human monocytes in a concentration-dependent manner. Scalaradial from 3 to 10 μM inhibited PGE_2 and TNFα production (data not shown). At 3 μM, scalaradial had little effect on the expression of PGHS-2, whereas at 10 μM it significantly inhibited PGHS-2 expression (Fig. 8, middle panel). Cell viability was not compromised by these concentrations of scalaradial (>95% in 5% FBS or >85% in 1% HIAS). Dexamethasone (1 μM) inhibited PGE_2 and TNFα production (data not shown), and inhibited PGHS-2 expression to basal levels. IL-1ra at a 10-fold molar excess over IL-1β reduced IL-1β-induced PGE_2 and TNFα synthesis (data not shown) as well as PGHS-2 expression (Fig. 8, middle panel). WEB-2086 at 30 μM had no effect on IL-1β-induced PGHS-2 expression (Fig. 8, upper panel), but significantly inhibited TNFα production (data not shown). The 5-lipoxygenase/cyclooxygenase inhibitor and cytokine release inhibitor SKF-86002 inhibited endogenous PGE_2 and TNFα production (data not shown),

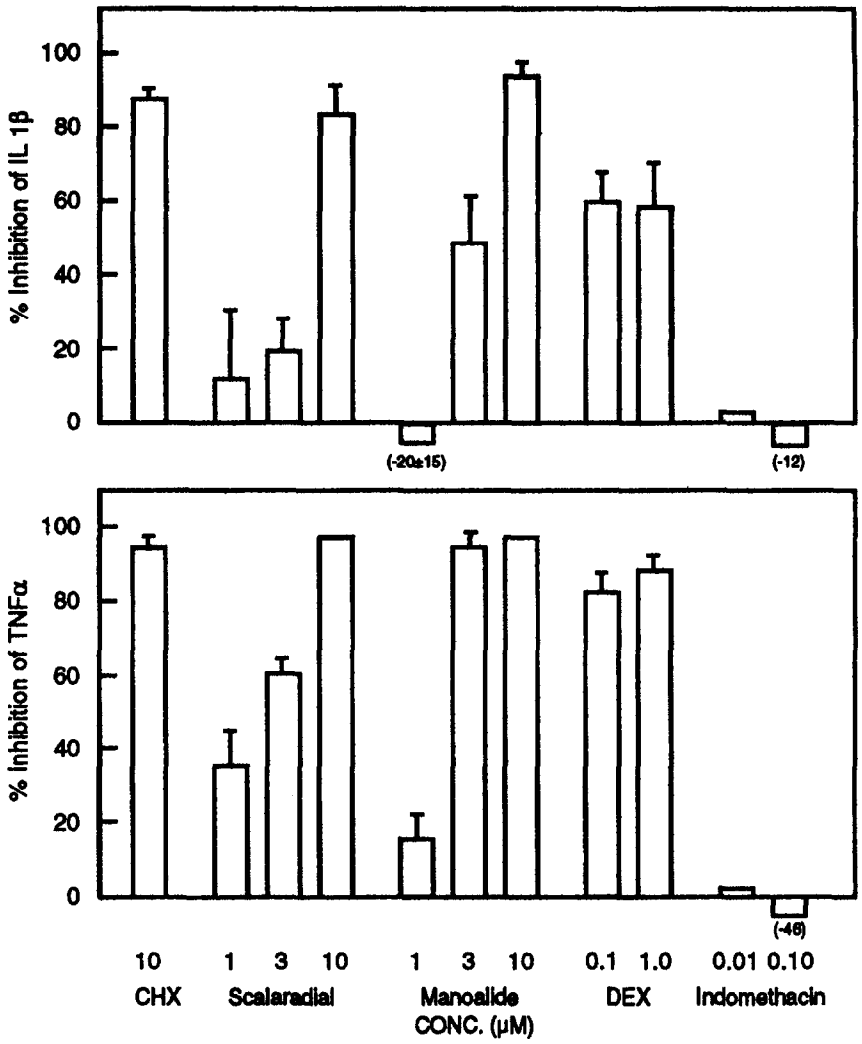


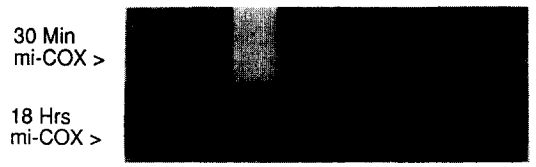
Fig. 4. Effects of inhibitors on the release of IL-1β and TNFα from LPS-treated monocytes. Monocytes were treated with various inhibitors for 18 hr in the presence of LPS (10 ng/mL) and 5% FBS. Released IL-1β and TNFα were measured in the culture medium by ELISA. Abbreviations: CHX, cyclohexamide; and DEX, dexamethasone. Values are means ± SEM, N = 3 donors. Total IL-1β release ranged from 0.75 to 5.23 ng/10^7 cells and TNFα release ranged from 0.41 to 10.25 ng/10^7 cells.

as well as PGHS-2 expression in LPS-stimulated monocytes (Fig. 6). However, in IL-1β-stimulated monocytes, SKF-86002 did not inhibit the expression of PGHS-2 (Fig. 6, upper panel). As demonstrated for LPS-stimulated monocytes, IL-1β-stimulated expression of PGHS-2 was not affected significantly by rolipram, zileuton (A64077), or indomethacin (Fig. 8, bottom panel).

DISCUSSION

PGHS is the rate-limiting enzyme in the prostaglandin biosynthesis pathway converting free arachidonic acid released by PLA_2 into numerous pro-inflammatory prostanoids. PGHS has long been the target for NSAIDs in the treatment of acute and chronic inflammatory diseases. Recently, a second form of PGHS was discovered in many tissues and cell types [8, 10, 11, 17]. This inducible form of PGHS (Cox-2, mi-COX, PGHS-2) is thought to be responsible for pathological prostaglandin biosynthesis, whereas the constitutive form of the enzyme, PGHS-1, is believed responsible for physiological or homeostatic prostaglandin formation [5, 19]. PGHS-2 is a very attractive isozyme for therapeutic intervention, and the design of selective inhibitors may be used to elucidate the role of PGHS-1 and PGHS-2 in the associated unwanted side-effects of currently available NSAIDs [5, 18, 19].

In this study, we evaluated some of the known inhibitors of the arachidonic acid metabolism pathway to determine their effect on PGHS-2 expression and eicosanoid biosynthesis in purified human monocytes. Two major classes of drugs have been used clinically to inhibit prostanoid biosynthesis—glucocorticoids and NSAIDs. Glucocorticoids have been shown to reduce prostaglandin synthesis in human fibroblasts [2, 23] and endothelial cells [14, 32] and various other cells [27]. In contrast, in other cell types such as amnion cells [33], glucocorticoids have been observed to increase prostanoid synthesis. Dexamethasone has been shown to in-



LPS	–	+	+	+	+	+	+	+	+
CHX (10μM)			+						
MLD (μM)	1	3	10						
SLD (μM)				1	3	10			
DEX (μM)							0.1		

Fig. 5. Effects of various inhibitors on the expression of PGHS-2 (mi-COX) in LPS-treated monocytes. Monocytes were treated with LPS for 30 min or 18 hr in the presence of inhibitor and 5% FBS. PGHS-2 (mi-COX) was identified by immunoblot after SDS–PAGE of equivalent amounts of protein added to each well. Abbreviations: CHX, cyclohexamide; DEX, dexamethasone; MLD, manoalide; and SLD, scalaradial. Data are representative of at least two experiments (donors) showing similar results.

hibit PGHS expression in IL-1β-induced fibroblasts [6] and LPS-induced alveolar macrophages [27]. We have also demonstrated that dexamethasone can inhibit endogenous PGE_2 synthesis and PGHS-2 expression to basal levels in purified human monocytes [20]. Western analyses of PGHS-2 protein levels and activity measurements with exogenous arachidonic acid suggest that dexamethasone as well as cyclohexamide, scalaradial, and manoalide only affect the induction/expression of the inducible form of PGHS. Previous hypotheses on the

LPS 10 ng/ml	–	+	+	+	+	+	+	
SKF86002 3μM	–	–	+	–	–	–	–	
SKF86002 10μM	–	–	–	+	–	–	–	
ILIRA 2 μg/ml	–	–	–	–	+	–	–	
A64077 5μM	–	–	–	–	–	+	–	
WEB 2086 10μM	–	–	–	–	–	–	+	

LPS 10 ng/ml	–	+	+	+	+	+	+	+
Rolipram 1 μM	–	–	+	–	–	–	–	–
Rolipram 3 μM	–	–	–	+	–	–	–	–
WEB2086 3 μM	–	–	–	–	+	–	–	–
WEB2086 10 μM	–	–	–	–	–	+	–	–
NDGA 3 μM	–	–	–	–	–	–	+	–
NDGA 10 μM	–	–	–	–	–	–	–	+

Fig. 6. Effects of various inhibitors on LPS-induced PGHS-2 expression. Human monocytes were treated with LPS for 18 hr in the presence of various inhibitors and 1% HIAS (heat-inactivated autologous serum). Immunoblots were performed as described in Materials and Methods. NDGA = nordihydroguaiaretic acid. All immunoblots are representative of at least two experiments (donors) showing similar results.

mechanism of dexamethasone postulated the induced synthesis of lipocortin and the subsequent inhibition of PLA_2 resulting in the inhibition of prostanoid biosynthesis [34]. However, this concept seems to be unsupported, and recent evidence suggests that the glucocorticoid mechanism involves the direct modulation of mRNA levels (induction and expression) of multiple inflammatory mediators (IL-1β and TNFα) and enzymes including PGHS-2 [25]. It is still not known whether the regulation of PGHS-2 expression is at the level of transcription or translation [6]. Recent studies would suggest an effect on transcription of the PGHS-2 gene in 3T3 fibroblasts [22] or on the translation of PGHS-2 mRNA in murine and chicken fibroblasts [23].

Previous studies have demonstrated that monokines, such as IL-1β, can induced PGHS-2 in human fibroblasts [2] and endothelial cells [14]. In our studies, we have demonstrated that IL-1β can induce the expression of PGHS-2, obtaining similar expression levels with endotoxin (LPS) as with high concentrations of IL-1β (>50 ng/mL) in purified human monocytes. In general, PGHS activity measured by application of exogenous arachidonic acid was only 1.5- to 2-fold greater than control, which agrees with the findings from other investigators [7, 8]. We further demonstrated that IL-1ra, a competitive inhibitor of the IL-1β receptor [30, 31], can reduce PGHS-2 expression as well as PGE_2 biosynthesis in both LPS- and IL-1β-stimulated monocytes. However, we were not able to stimulate PGHS-2 expression with TNFα (data not shown). Since TNFα fails to induce PGHS-2 activity, it seems that TNFα does not modulate PGHS-2 expression even though IL-1β and TNFα have similar physiological effects in many cell types. To further test this hypothesis, we used rolipram, a cAMP-dependent phosphodiesterase IV inhibitor, which selectively inhibits TNFα production in LPS-stimulated human monocytes [35]. Rolipram had no significant effect on PGE_2 biosynthesis, PGHS-2 expression or IL-1β synthesis, but significantly inhibited TNFα production. These data support the role of IL-1β and not TNFα in the induction and expression of the PGHS-2 gene in the human monocyte.

Manoalide and scalaradial exhibit the majority of their anti-inflammatory effects through the inhibition of 14 kDa PLA_2 ($sPLA_2$) [36, 37]. However, in acute ionomycin/PMA-stimulated eicosanoid production (30 min), neither manoalide nor scalaradial was able to inhibit PGE_2 production, whereas LTB_4 and PAF production were inhibited, as expected. Interestingly, both manoalide and scalaradial inhibited prostaglandin synthesis after 18-hr preincubation with the monocytes and subsequent stimulation with ionomycin/PMA. This result suggested that these marine natural products may have an unusual effect on prostaglandin-forming enzymes, which manifests itself only after prolonged incubation (i.e. at the protein synthesis level). Data from western analyses showed that scalaradial can effectively inhibit both LPS- and IL-1β-induced PGHS-2 expression. Since scalaradial also inhibits IL-1β-induced PGHS-2 expression, it would suggest an effect of scalaradial on the transcriptional machinery regulating PGHS-2 induction. Manoalide is not as effective as scalaradial in the regulation of LPS-induced PGHS-2 expression, and it is less effective against IL-1β-induced PGHS-2 synthesis. Considering their similar *in vitro* profiles and properties, these data suggest that these two marine natural products

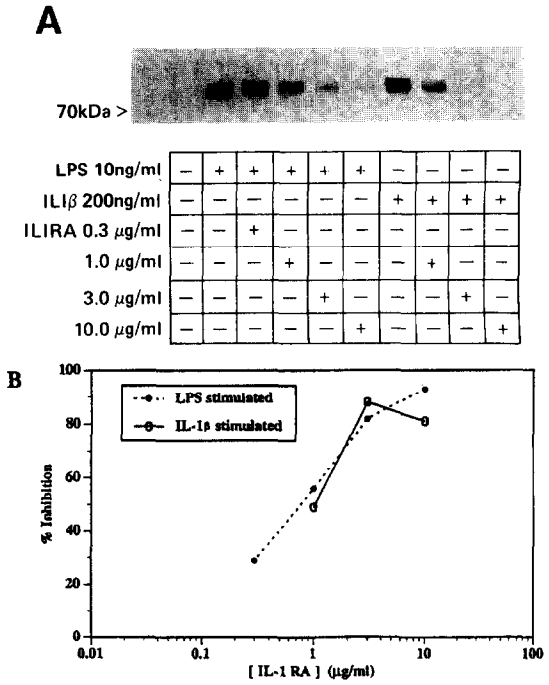


Fig. 7. Effects of IL-1ra on LPS-induced PGHS-2 expression. (A) Concentration–response study with IL-1ra (0.3 to 10 μg/mL) in the presence of LPS (10 ng/mL) for 18 hr. (B) Western analyses digitized and converted to arbitrary units to demonstrate concentration–response relationship (apparent IC_{50} = 0.8 μg/mL). Data are representative of at least two experiments (donors) showing similar results.

may be acting differently in the pathways resulting in PGHS-2 expression. We also pretreated monocytes with aspirin prior to LPS or IL-1β stimulation and observed no significant difference on PGHS-2 expression or prostaglandin biosynthesis in human monocytes. In contrast, in cultured human umbilical vein endothelial cells, aspirin has been shown to inhibit IL-1-induced PGHS expression [38]. Since the induction of PGHS mRNA varies in different cells, it is not certain whether the inhibition by aspirin is due to effects on PGHS-1 or PGHS-2 expression; however, these data suggest some regulation of PGHS-1 in endothelial cells.

Inhibition of PAF or leukotriene synthesis does not affect PGHS-2 expression, as demonstrated by the use of WEB-2086 and zileuton. These results suggest that PAF and LTB_4/LTC_4 are probably not involved in the regulation of the signal transduction process involved in either LPS- or IL-1β-induced *de novo* synthesis of PGHS (specifically PGHS-2). Coupled with the lack of effect of indomethacin and aspirin (PGHS inhibitors) on the expression of PGHS-2, these data suggest that eicosanoid products are not involved in the negative or positive regulation of PGHS-2 expression in human monocytes. However, this does not leave out the possibility that a novel eicosanoid or arachidonic acid itself may be playing some role. Protein tyrosine kinase activity has been shown to regulate eicosanoid biosynthesis in resident mouse peritoneal macrophages [39] and prostaglandin synthesis in LPS-primed $P388D_1$ macrophage-like cells [40]. Genistein and tyrphostin 25 can effectively inhibit PGHS-2 expression and PGE_2 production (unpublished observations), suggesting that tyrosine phosphorylation may play an important role in LPS signal transduction [41] and subsequent PGHS-2 expression [42]. Figure 9 presents the major events that may occur when the human monocyte is activated by LPS or ionophore/PMA. Little is known about the mechanisms controlling PGHS gene transcription in human cells. Two major signal transduction mechanisms appear to be involved in PGHS expression: one involves the activation of protein kinase C (PKC) [4, 43], and the other may involve tyrosine phosphorylation events through the LPS receptor [41, 44]. Ionophore/PMA involves the direct influx of Ca^{2+} and the activation of PKC and mitogen-activated protein kinases, which stimulate $cPLA_2$ and release arachidonic acid for prostaglandin synthesis. LPS signal transduction may involve protein tyrosine kinase activation without the mobilization of extracellular Ca^{2+} (explaining the lack of leukotriene production with LPS stimulation) and the direct regulation of transcription factors that regulate cytokine synthesis. Since IL-1β (200 ng/mL) produces similar levels of PGHS activation

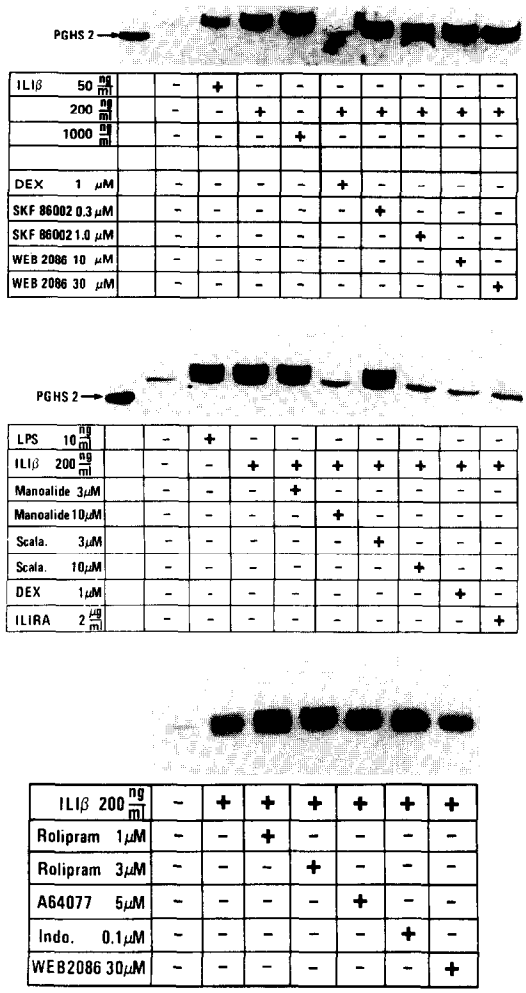


Fig. 8. IL-1β-induced PGHS-2 expression and effects of various inhibitors on IL-1β-induced PGHS-2 expression. IL-1β was added to human monocytes for 18 hr at 37° in the presence of 1% HIAS. Immunoblots for PGHS-2 were performed as described for LPS-treated cells. Abbreviations: DEX, dexamethasone; Scala., scalaradial; IL1RA, IL-1ra; and Indo., indomethacin. All immunoblots are representative of at least two experiments (donors) showing similar results.

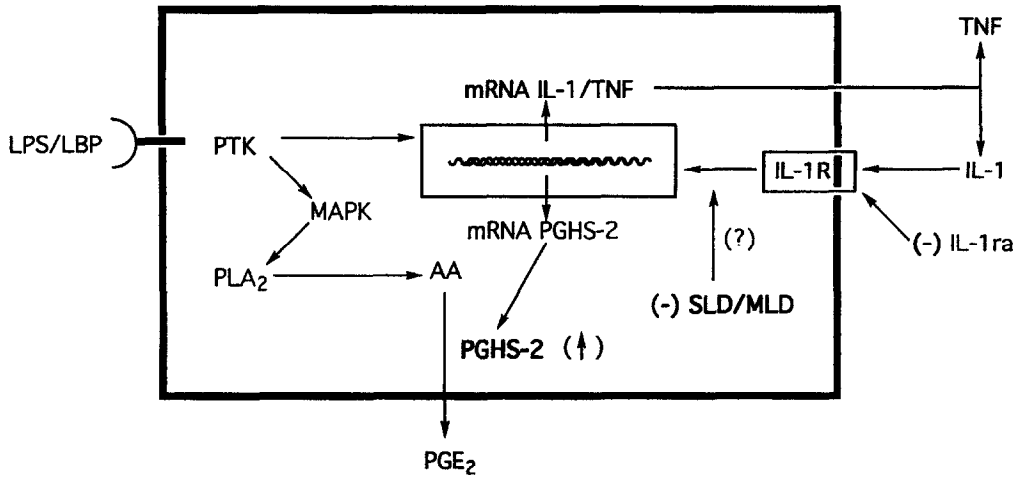


Fig. 9. Human monocyte activation via LPS. Shown is a schematic diagram of the human monocyte demonstrating the multiple pathways available to generate eicosanoids. IL-1β seems to be the predominant cytokine involved in the up-regulation of PGHS-2 (COX-2). Abbreviations: PLA_2, phospholipase A_2; PTK, protein tyrosine kinase; MAPK, mitogen-activated protein kinases; LBP, LPS binding protein; SLD, scalaradial; and MLD, manoalide.

as LPS (10 ng/mL), IL-1β may be the effector of LPS-induced PGHS gene expression. The genomic clones for human PGHS have been made available recently [13, 14, 17]. This will give some insight into how this intriguing enzyme is regulated at the molecular level and clues to designing inhibitors for this particular cyclooxygenase (PGHS) isozyme.

The discovery of the effects of manoalide and scalaradial on PGHS-2 expression led to a study on the regulation of PGHS-2 expression in the human monocyte. Since these marine natural products are effective inhibitors of 14 kDa PLA_2 ($sPLA_2$) *in vitro*, we examined their possible effects on the regulation of PGHS-2 expression through eicosanoids (PAF, PGs, LTs). From our studies one could conclude that the effects of scalaradial and manoalide on PGHS-2 expression are independent of any effect on lipid mediator production. Through the use of selective inhibitors of cytokine release, IL-1β seems to be the major inducer of PGHS-2 in LPS-stimulated monocytes. Interestingly, a difference in the effects of manoalide and scalaradial can be seen with IL-1β induction, that is scalaradial is still able to block PGHS-2 synthesis. Though their mechanisms *in vitro* may be similar [45], they appear to have unique and interesting activities in the intact cell, which may potentiate their apparent anti-inflammatory effects.

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