Lipopolysaccharide Induces Prostaglandin H Synthase-2 Protein and mRNA in Human Alveolar Macrophages and Blood Monocytes

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Abstract

We and others have previously demonstrated that human alveolar macrophages produce more PGE_2 in response to lipopolysaccharide (LPS) than do blood monocytes. We hypothesized that this observation was due to a greater increase in prostaglandin H synthase-2 (PGHS-2) enzyme mass in the macrophage compared to the monocyte. To evaluate this hypothesis, alveolar macrophages and blood monocytes were obtained from healthy nonsmoking volunteers. The cells were cultured in the presence of 0 to 10 μg/ml LPS. LPS induced the synthesis of large amounts of a new 75-kD protein in human alveolar macrophages, and a lesser amount in monocytes. Synthesis of this protein required more than 6 h and peaked in 24 to 48 h; the protein reacted with an anti–PGHS-2 antibody prepared against mouse PGHS-2. Associated with synthesis of the protein was a marked increase in LPS-stimulated and arachidonic acid-stimulated synthesis of PGE_2 by alveolar macrophages compared to monocytes. Cells not exposed to LPS contained only PGHS-1 and synthesized very little PGE_2 during culture or in response to exogenous arachidonic acid. An LPS-induced mRNA, which hybridized to a human cDNA probe for PGHS-2 mRNA, was produced in parallel with production of this new protein and was produced in much greater amounts by alveolar macrophages compared to blood monocytes. This mRNA was not detectable in cells not exposed to LPS. In contrast, both types of cells contain mRNA, which hybridizes to a cDNA probe for PGHS-1. This mRNA did not increase in response to LPS. LPS also had no effect on PGHS-1 protein. These data demonstrate that PGE_2 synthesis in human alveolar macrophages and blood monocytes correlates to the mass of PGHS-2 in the cell. We conclude that the greater ability of the macrophage to synthesize PGE_2 in response to LPS is due to greater synthesis of PGHS-2 by the macrophage. (*J. Clin. Invest.* 1994. 93:391–396.) Key words: prostaglandin synthase • prostaglandins E

Introduction

The alveolar macrophage is the predominant immune and inflammatory effector cell of the human lung. Although it can initiate inflammation, it also has the capacity to suppress inflammation. In response to the inflammatory mediator lipopolysaccharide (LPS), the macrophage will generate large amounts of the anti-inflammatory prostaglandin, prostaglandin E_2 (PGE_2) (1, 2). We previously demonstrated that LPS stimulates the macrophage to produce increased amounts of prostaglandin H synthase (PGHS)[1] (3). This increase in PGHS mass is believed to markedly increase the capacity of the macrophage to generate PGE_2 (3).

It is now evident that in many cell lines there exist two forms of PGH synthase: a constitutive enzyme, designated PGHS-1; and an inducible enzyme, PGHS-2, which has a unique message (4, 5, 6, 7), a unique amino acid sequence (7, 8), and a unique activity profile (9). We wished to determine if the LPS-stimulated increase in human macrophage PGHS and PGE_2 formation was due to synthesis of PGHS-2 as suggested by recent studies in rat alveolar macrophages (10), or due to synthesis of PGHS-1 as suggested by studies on rat peritoneal and alveolar macrophages (11). In addition, we wanted to explore whether the increased ability of the alveolar macrophage, compared to the peripheral blood monocyte, to synthesize PGE_2 following LPS exposure was due to increased synthesis of PGHS-2 by the alveolar macrophage (12).

Methods

Isolation of alveolar macrophages. The study was approved by the Committee for Investigations Involving Human Subjects at The University of Iowa. To obtain human alveolar macrophages, normal volunteers with a lifetime nonsmoking history, who had no acute or chronic medical illness and were on no medications, underwent bronchoalveolar lavage, as previously described (13). The lavage procedure used five 25-ml aliquots of sterile, warmed saline in each of three segments of the lung.

The lavage fluid was filtered through two layers of gauze and centrifuged at 1,500 rpm for 5 min. The cell pellet was washed twice in HBSS without Ca^{2+} or Mg^{2+} and was suspended in culture medium as described below. Cell counts were determined by an electronic counter (Coulter Electronics, Hialeah, FL). Cell differentials were generated by Wright–Giemsa-stained cytocentrifuge preparations.

Isolation of blood monocytes. 225 ml of heparinized blood were obtained by venipuncture following bronchoscopy. Mononuclear cells were isolated using a Ficoll-Hypaque gradient (14). Monocytes were isolated from the mononuclear fraction by washing off the non-adherent cells after incubation for 1 h at 37°C. Cell counts and differentials were performed as above.

Cell culture. The macrophages and monocytes were cultured at a density of 1×10^6 cells/ml in Rosewell Park Memorial Institute tissue culture medium (RPMI 1640) containing 0.3 mg/ml L-glutamine, 80 μg/ml gentamicin, and 5% endotoxin-free FCS (Hyclone Laboratories, Logan, UT). 6-well flat polystyrene culture dishes (Costar, Cambridge, MA), containing 4 ml of suspended cells in each well, were incubated in an atmosphere of 95% humidified air–5% CO_2 at 37°C.

1. *Abbreviation used in this paper:* PGHS, prostaglandin H synthase.

Control and lipopolysaccharide (LPS; *Escherichia coli* 026:B6, Sigma Chemical Co., St. Louis, MO) cultures were set up as described in the text and figure legends. At the end of LPS exposure the cell supernatants were removed. The supernatants and the cells were stored frozen at −70°C. Viability of the cultured cells was determined using trypan blue exclusion.

Antibody preparation. An in-house polyclonal rabbit anti–PGHS antibody prepared against purified ram seminal vesicle PGHS was used where described in the text (3). Preliminary studies demonstrated that this antibody will detect ≤ 250 ng PGHS-1, purified from ram seminal vesicles, and ≤ 250 ng chicken-recombinant PGHS-2 (nos. PG01 and PG04; Oxford Biomedical Research, Inc, Oxford, MI).

The anti–PGHS-2 was a polyclonal rabbit anti–mouse antibody (no. PG26, Oxford Biomedical Research, Inc.) that was found to be highly specific. The antibody does not cross-react with PGHS-1 nor does it cross-react with a panel of molecular weight marker proteins, and it has no apparent cross-reactivity against other human cell proteins.

Immunoblot detection of PGHS-1 and PGHS-2. Cell pellets from alveolar macrophages and blood monocytes were lysed in 150 µl of solubilization buffer at 25°C (1% Tween 20, 10 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 171 µg/ml diethyldithiocarbamic acid, 200 µg/ml α2-macroglobulin, and 50 mM Tris, pH 8, all from Boehringer Mannheim Biochemicals, Indianapolis, IN). The cell material was sonicated for 15 s and then centrifuged at 15,000 g. An aliquot of the supernatant was used to determine protein by the Coomassie blue method (15). Supernatants were then mixed 1:1 with sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue, and 1.25 M Tris, ph 6.8). Equal amounts of protein (50–100 µg) were then loaded onto a Bio-Rad Mini-electrophoresis polyacrylamide gel system (Bio-Rad Laboratories, Richmond, CA) using a 4% stack and a 10% running gel, and then run at 80 v for 3 h. Cell proteins were transfered to nitrocellulose overnight at 30 v. The nitrocellulose was then blocked with 5% skim milk, washed, and then incubated with antibody for 1 h at 25°C. The polyclonal in-house anti–PGHS antibody was diluted 1:5,000 and the commercial anti–PGHS-2 antibody was diluted 1:200. The Amersham ECL system was used to detect the antibodies. Kodak X-omat AR film with cassette closure times of 10 to 60 s resulted in adequate exposure to visualize the bands.

Northern blot analysis. RNA was isolated from the cells by the guanidine isothiocyanate-CsCl gradient procedure (16). The isolated RNA was fractionated in a 1.5% denaturing agarose gel containing 2.2 M formaldehyde (17). *E. coli* 23S (3.3 kb) and 16S (1.7 kb) rRNAs were included as molecular size standards. RNA loading was confirmed by equivalent ethidium bromide staining in each lane. The RNAs were then transferred to GeneScreen Plus paper as suggested by the manufacturer. PGHS-1 and PGHS-2 cDNA probes (Oxford Biomedical Research, Inc.) were ^{32}P-labeled by nick translation, then hybridized to the blots at 42°C. Autoradiograms were prepared using exposure times of 18 h.

PGE$_2$ determination. PGE$_2$ was measured using a commercial ELISA kit (Oxford Biomedical Research, Inc.).

Statistics. All results shown were confirmed in at least two additional experiments. Linear data were analyzed by one- or two-way ANOVA. Categorical data were analyzed by unpaired *t* statistics. The specific tests used and the results are depicted in the figure legends.

Results

Preliminary studies comparing human alveolar macrophages to peripheral blood monocytes suggested that LPS caused an increase in the molecular weight of PGHS in the macrophage. To clarify this observation, a large gel electrophoresis of cell proteins from monocytes and macrophages was performed, and then followed by Western blotting using a polyclonal antibody which detects both PGHS-1 and PGHS-2 (3). Fig. 1 demonstrates that following LPS two proteins are detected, one of
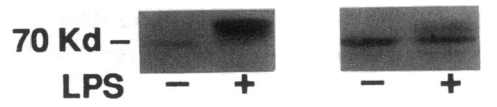

Macrophages Monocytes

Figure 1. Effect of LPS on PGHS enzyme mass in macrophages and monocytes. Macrophages and monocytes from the same subject were cultured 24 h in the absence (−) or presence (+) of 10 µg/ml LPS. 100 µg of cell protein was loaded onto a 14-cm SDS 7.5% polyacrylamide slab gel. Immunoblots were then performed with the in-house polyclonal antibody that detects both PGHS-1 and PGHS-2.

$\sim$ 70 kD, which does not increase with LPS stimulation, and a second protein of $\sim$ 75 kD, which is not present in the control cells. This new protein becomes the predominant protein detected in the alveolar macrophage following LPS, and is present in lesser amounts in the LPS-exposed monocytes.

To explore this finding further, macrophages and monocytes were exposed to 10 µg/ml LPS for periods of 6 to 48 h. LPS caused a time-dependent increase in the 75-kD protein in both monocytes and macrophages that was maximum at 24–48 h. An immunoblot from the macrophages is shown in Fig. 2 *A*. There was also a time-dependent increase of PGE$_2$ formation from the LPS-exposed cells that paralleled the increase in the 75-kD protein as shown in Fig. 2 *B*. These results demonstrate that LPS increases prostaglandin synthesis and increases
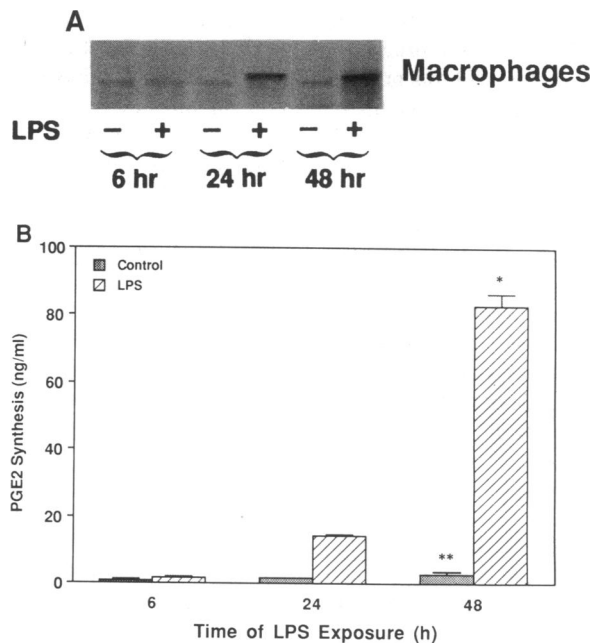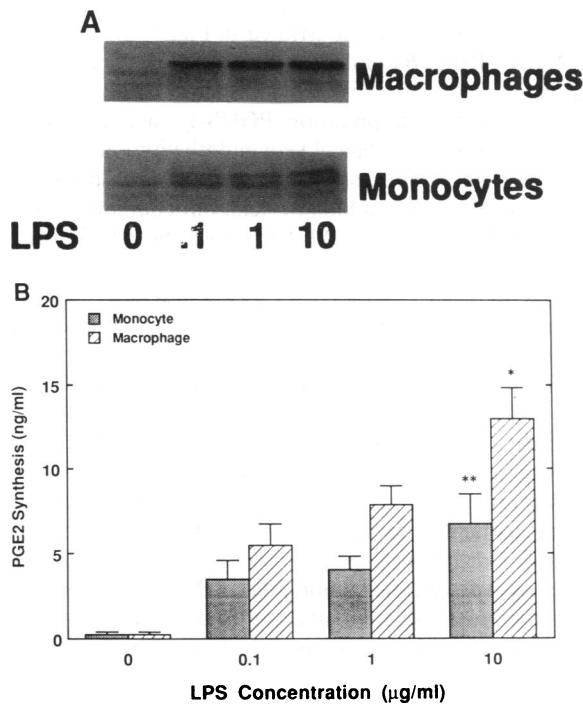

Figure 2. Effect of time of exposure to LPS on PGHS enzyme mass and PGE$_2$ synthesis. (*A*) Macrophages were cultured 6, 24, and 48 h with 10 µg/ml LPS. Immunoblots were then performed with the in-house polyclonal antibody that detects both PGHS-1 and PGHS-2. (*B*) Aliquots of the medium from the cells shown in *A* were assayed for PGE$_2$ by ELISA. Error bars are±SEM, *n* = 3. The increase in PGE$_2$ synthesis by LPS-exposed macrophages is significant by the Kruskal–Wallace one-way ANOVA, *P < 0.05. In addition, the difference in PGE$_2$ formation between Control and LPS macrophages is significant by two-way ANOVA, **P < 0.005.
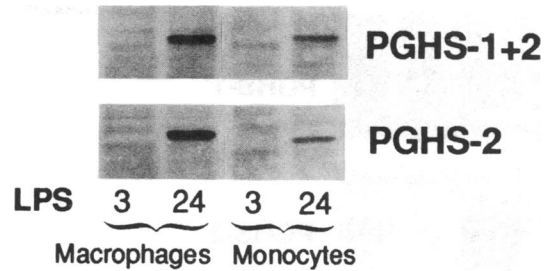
the amount of a 75-kD protein in cultured monocytes and macrophages, consistent with previous observations (3).

Fig. 3 depicts the increase in amount of the 75-kD protein in cultured monocytes and macrophages in response to LPS concentrations of 0.1 to 10 μg/ml for 24 h (A). The results shown demonstrate that the macrophage responds to lower concentrations of LPS than the monocyte. PGE_2 synthesis increases concurrent with the increase in the 75-kD protein as shown in Fig. 3 B. These observations indicate that there is a LPS concentration-dependent increase in the 75-kD protein mass in monocytes and macrophages that is associated with increased PGE_2 formation.

These findings raised the question whether the higher molecular weight LPS-inducible protein was the recently described mitogen-inducible PGHS-2. To address this, LPS-exposed macrophages and monocytes were lysed and cell proteins were evaluated by Western analysis with a specific anti–PGHS-2 antibody. The immunoblot shown in Fig. 4 demonstrates that only the new 75-kD band is detected by the specific antibody. The blot also shows that cells exposed to 3 h of LPS do not yet express this protein (3). The immunoblot in Fig. 5 compares the anti–PGHS-2 antibody (antibody B) to the
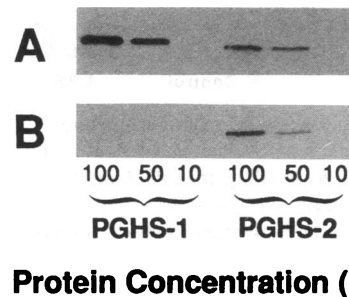

Figure 4. Detection of PGHS-2 in monocytes and macrophages. Macrophages and monocytes were cultured 3 or 24 h in the presence of 10 μg/ml LPS. Cell proteins were then immunoblotted with the in-house antibody (PGHS-1 + 2) or the antibody specific for PGHS-2 (PGHS-2).

in-house antibody (antibody A). Fig. 5 demonstrates that the anti–PGHS-2 antibody detects only PGHS-2. These findings support the hypothesis that the new protein is PGHS-2.

Based on the observations of other investigators (5, 6, 7, 18), it seemed likely that appearance of PGHS-2 protein would require an increase in PGHS-2 mRNA. To explore this question, Northern blotting of human macrophage and monocyte RNA was performed following incubation with human cDNA probes for PGHS-1 and PGHS-2. These studies, shown in Fig. 6, demonstrate that the PGHS-2 cDNA probe detects an approximately 5-kb message in macrophages and, to a lesser degree, in monocytes exposed to LPS (7). The message detected by the PGHS-1 cDNA, also $\sim$ 5 kb, did not change (7). These findings show that LPS causes an increase in PGHS-2 message in human macrophages and monocytes. In addition, the findings strengthen our hypothesis that the new protein is PGHS-2.

We speculated that the increase in PGHS-2 mass induced by LPS resulted in the increase in PGE_2 synthesis. However, if PGHS-2 were an inactive protein, prostaglandin synthesis might still increase if LPS caused higher phospholipase A_2 activity, making more substrate available for PGHS-1 (11, 19, 20, 21). To explore this possibility, 24 h of LPS stimulated macrophages and monocytes were washed with buffer and then exposed to 20 μM arachidonic acid for 15 min. If the newly synthesized PGHS-2 were active, then the LPS-exposed cells would make more PGE_2 than controls when stimulated with extracellular arachidonic acid. Fig. 7 demonstrates that control cells make little PGE_2 in the basal state (graph A) and undetect-

Figure 3. Effect of concentration of LPS on PGHS enzyme mass and PGE_2 synthesis. (A) Monocytes and macrophages were cultured 24 h with 0 to 10 μg/ml LPS. Immunoblots were then performed with the in-house polyclonal antibody that detects both PGHS-1 and PGHS-2. Computerized image analysis of the higher molecular mass band (75 kD) revealed an integrated optical density of 0, 950, 1,424, and 1,537 for the macrophage protein and 50, 168, 263, and 726 for the monocyte protein after LPS concentrations of 0, 0.1, 1, and 10 μg/ml respectively. (B) Aliquots of the medium from the cells shown in A were assayed for PGE_2 by ELISA. Error bars are±SEM. Results are from three different subjects. The increase in PGE_2 synthesis by macrophages is significant by the Kruskal–Wallace one-way ANOVA, *$P < 0.05$. In addition, the difference in PGE_2 formation by monocytes and macrophages is significant by two-way ANOVA, **$P < 0.025$.

Figure 5. Sensitivity and specificity of antibodies to PGHS-1 and PGHS-2 protein. Our in-house antibody (A), and an antibody specific for PGHS-2 (B), were immunoblotted against purified ram seminal vesicle PGHS-1 (70 kD) and a recombinant chicken PGHS-2 (69 kD). Antibody A was diluted 1:5,000. Antibody B was diluted 1:200. The quantity, in nanograms, of PGHS-1 and PGHS-2 protein is shown at the bottom of the figure. Note that the recombinant chicken PGHS-2, shown above, has a lower molecular weight than the human protein.

PGHS-1

PGHS-2

LPS   −   +   −   +

Macrophages   Monocytes
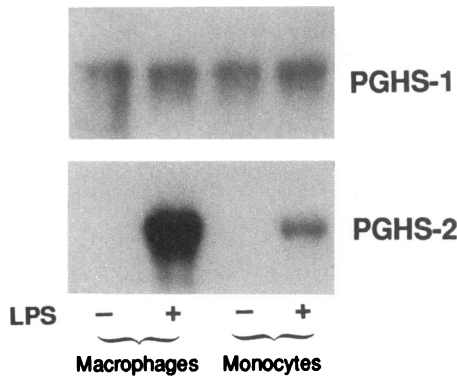
Figure 6. Effect of LPS on PGHS-1 and PGHS-2 mRNA. Macrophages and monocytes were cultured 24 h in the absence (−) or presence (+) of 10 μg/ml LPS. Northern analysis was then performed using ^{32}P-labeled human cDNA probes specific for PGHS-1 mRNA (PGHS-1) and PGHS-2 mRNA (PGHS-2).

able amounts when exposed to arachidonic acid (graph *B*). This demonstrates that cells not exposed to LPS have limited ability to generate PGE$_2$, indicating that the constitutive enzyme, PGHS-1, has low activity. However, the LPS-exposed cells generate PGE$_2$ in the 24-h culture medium (*graph A*) and immediately synthesize PGE$_2$ when exogenous arachidonic acid is added (*graph B*). In both cases the macrophages generate more PGE$_2$ than the monocytes. These results support the conclusion that PGHS-2 is the primary cyclooxygenase in macrophages and monocytes after LPS stimulation. In addition, these observations indicate that synthesis of PGHS-2 is required for macrophages and monocytes to increase their capacity to generate PGE$_2$ from arachidonic acid.
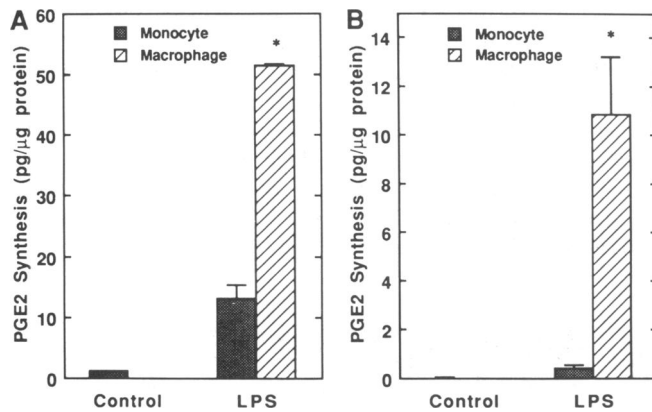
Figure 7. Effect of LPS on PGE$_2$ synthesis by macrophages and monocytes. Macrophages and monocytes were cultured 24 h in the absence (*Control*) or presence (*LPS*) of 10 μg/ml LPS. Graph (*A*) shows PGE$_2$ released into the medium over 24 h. Error bars are ±SEM, $n = 3$. The difference in PGE$_2$ synthesis between monocytes and macrophages exposed to LPS was statistically significant, *$P < 0.0005$ by the unpaired t statistic. Graph (*B*) shows PGE$_2$ synthesized after the cells used in graph *A* were washed and then stimulated with 20 μM arachidonic acid for 15 min. The difference in PGE$_2$ formation between LPS-exposed monocytes and macrophages stimulated with arachidonic acid was statistically significant, *$P < 0.002$ by the unpaired t statistic. Note that the magnitude of the y-axis is smaller in graph *B*.

Discussion

These studies demonstrate that LPS stimulates production of large amounts of PGHS-2 in human alveolar macrophages and smaller amounts of this protein in peripheral blood monocytes. The increase in PGHS-2 mass is associated with increased formation of PGE$_2$. The constitutive cyclooxygenase, PGHS-1, appears to have very low activity. This implies that both of these cell types require synthesis of PGHS-2 before they are able to generate larger amounts of PGE$_2$ (10, 22). However, the human alveolar macrophage has greater capacity to synthesize PGE$_2$, demonstrating an important difference between the resident airway macrophage and the peripheral blood monocyte.

The observation that PGHS-1 does not generate prostaglandin after exposure to exogenous arachidonic acid as shown in Fig. 7, does not prove that the constitutive enzyme is structurally inactive. It may be that PGHS-1 has been previously inactivated by generating prostaglandin from endogenous arachidonic acid (23). In that case, new enzyme synthesis would be required for PGHS-1 to demonstrate activity. If the turn-over of PGHS-1 in alveolar macrophages is slow, then at any one time there would be very little active enzyme in the cell. This seems the most likely explanation for the apparent inactivity of the enzyme demonstrated in Fig. 7, since the results in Fig. 2 (Panel *B*) indicate a small basal level of PGE$_2$ generation.

In support of this interpretation, PGHS-1 is active in other cells (24, 25). Human umbilical vein endothelium, for example, produces large amounts of prostaglandin in response to thrombin or exogenous arachidonic acid, but contains almost exclusively PGHS-1 (our unpublished observations). This tissue has slow turnover of functional enzyme. Following inactivation of endothelial PGHS-1 with excess arachidonic acid, H$_2$O$_2$, reoxygenation, or aspirin, 6 to 48 h is required for return of PGI$_2$ synthesis, even though prostacyclin synthase remains active (26, 27, 28, 29, 30), implying a slow turnover of PGHS-1. These functional observations differ from the much faster turnover rates calculated by pulse–chase methods (31, 32).

The peripheral blood monocyte, believed to be the primary source of human alveolar macrophages, migrates from the blood compartment to become a resident of the lung airspace (33, 34, 35). During this process the cell increases in size, acquires increased phagocytic, tumoricidal, and microbicidal abilities, and becomes able to synthesize lipoxygenase products (36, 37, 38, 39, 40). In addition, as demonstrated by the experiments in this study, the cell also acquires an increased capacity to synthesize the protein PGHS-2 and its product PGE$_2$.

The acquired ability of the macrophage to synthesize more PGE$_2$ than the blood monocyte may be protective of the human lung. PGE$_2$ has been demonstrated to inhibit IL-1 activity, PMA-induced lymphocyte proliferation, IL-8 synthesis, and synthesis of collagen by fibroblasts (1, 41, 42). It is also an effective bronchodilator. Alternatively, the lower capacity of the peripheral blood monocyte to generate PGE$_2$ may also have protective benefits to the host. For example, in the blood compartment inhibiting lymphocyte proliferation, neutrophil migration (an IL-8 function), and collagen synthesis may decrease the host's ability to control infection. In that case, less PGE$_2$ would be beneficial.

The discovery of PGHS-2 has greatly advanced our understanding of prostaglandin regulation. Previous observations of

increased PGE_2 synthesis after exposure of cells to v-*src*, IL-1, IL-2, TNF, PDGF, phorbol esters, cAMP, and LPS can now be explained by the ability of these agents to induce synthesis of PGHS-2 (4, 7, 20, 22, 43–47). The only known tissues, at the time of this writing, in which PGHS-2 mRNA is expressed primarily are prostate and neonatal thymus (48). In tissues in which PGHS-2 is expressed in response to an exogenous signal, such as LPS, it has characteristics of an early response gene (49).

In this study we demonstrate that human monocytes and macrophages have no detectable message for PGHS-2 under control conditions. After LPS there is appearance of an easily detectable ∼ 5-kb mRNA that binds the PGHS-2 cDNA probe. The amount of message is greater in macrophages, consistent with the findings of increased PGHS-2 protein and PGE_2 synthesis by these cells. These observations are similar to reports on other species and tissues, which demonstrate a single ∼ 4.5-kb mRNA binding PGHS-2 cDNA after LPS or mitogen exposure (5, 6, 7, 47, 50, 51). Our results differ from these reports in that we do not find a ∼ 2.8-kb message for PGHS-1. However, we do detect a message of ∼ 5 kb that binds the PGHS-1 cDNA probe. This larger message has been detected in other reports (7, 47, 50, 52) and is prominent in human tissues (7, 40, 52).

To confirm that our probe would detect the 2.8-kb message, we probed human dermal fibroblasts and detected both the 2.8- and the 5-kb messages. We then superinduced PGHS-2 with IL-1 and cycloheximide. The PGHS-2 probe detected a new 5-kb mRNA but the PGHS-1 2.8- and 5-kb bands did not change. These findings demonstrate that the PGHS-1 probe detects the 2.8-kb message and does not cross react with the PGHS-2 mRNA. At this time, we are unable to explain the absence of the 2.8-kb message in our cells.

The observations in this manuscript demonstrate the necessity of PGHS-2 for increasing synthesis of PGE_2 by the human alveolar macrophage and the peripheral blood monocyte. The observations we report are consistent with findings in rabbits and rats, which indicate that increased PGHS-2 mass correlates with increased PGE_2 synthesis in alveolar macrophages after LPS stimulation (6, 10). Our findings are in contrast to a primary role for PGHS-1 in macrophage prostaglandin synthesis, as suggested by recent studies on rat peritoneal and alveolar macrophages (11).

In summary, these observations demonstrate that, in humans, it is the inducible cyclooxygenase, PGHS-2, and not the constitutive enzyme, PGHS-1, that increases the capacity of the alveolar macrophage and the blood monocyte to generate prostaglandins after exposure to LPS.

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