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Concordant Induction of Prostaglandin E_2 Synthase with Cyclooxygenase-2 Leads to Preferred Production of Prostaglandin E_2 over Thromboxane and Prostaglandin D_2 in Lipopolysaccharide-Stimulated Rat Peritoneal Macrophages

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Rat peritoneal macrophages were stimulated with lipopolysaccaride (LPS) for various periods and their ability to convert exogenous arachidonic acid to various prostanoids was examined. Unstimulated cells, which expressed cyclooxygenase (COX)-1 but not COX-2, produced thromboxane (TX) B_2 > prostaglandin (PG) D_2 > PGE_2, whereas cells stimulated for 6–12 h with LPS exhibited marked increase in conversion to PGE_2, which paralleled COX-2 induction, with minimal change in conversion to TXB_2 and PGD_2. Pharmacological studies showed that formation of PGE_2 was mediated predominantly by COX-2, PGD_2 by COX-1, and TXB_2 by both COX-1 and COX-2 depending upon the timing of LPS stimulation. Measurement of the conversion of exogenous PGH_2 to each prostanoid in cell lysates demonstrated LPS-dependent increase in PGE_2 synthase activity that was degenerated by pretreatment with actinomycin D or cycloheximide. Thus, concordant induction of terminal PGE_2 synthase with COX-2 leads to the preferred production of PGE_2 to TXB_2 and PGD_2 by LPS-stimulated macrophages. © 1997 Academic Press

Cyclooxygenase (COX) catalyzes a key step in the biosynthesis of prostaglandins (PGs) by conversion of arachidonic acid, which is liberated from membrane phospholipids by phospholipase A_2, to PGH_2, an intermediate precursor of a variety of bioactive prostanoids (Reviewed in 1-3). Conversion of PGH_2 to each prostanoid is catalyzed by respective terminal prostanoid synthases varying in cell and tissue distribution and subcellular localization (4-12). Two isoforms of COX, COX-1 and COX-2, have approximately 60% sequence identity overall with much higher sequence conservation in their catalytic domains (1-3). COX-1 is constitutively expressed in most cells and tissues and is generally thought to serve certain physiologic housekeeping functions, whereas COX-2 is dramatically induced in response to a wide variety of stimuli, and is thought to contribute to the generation of PGs in certain stages of cell proliferation and differentiation and at sites of inflammation. It has been found recently that the two COX isoforms can be regulated separately even when both enzymes are present in the same cell (13-15). The absolute requirement for COX-2 in the delayed prostanoid generation, irrespective of the constitutive presence of COX-1, has been demonstrated by studies using COX-2-selective inhibitors (13), antisense (14), and knockout mice (15). Dependence of IgE-dependent immediate PGD_2 generation on COX-1, even when COX-2 was maximally induced, was demonstrated in mast cells (13). Although several possibilities have been proposed to explain the functional segregation of the two COX isoforms, such as their different subcellular localizations (16) and their different substrate concentration requirements (17), it is as yet largely obscure as to how each COX is selectively utilized in particular phases of cell activation.

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The abbreviations used are as follows: COX, cyclooxygenase; PLA_2, phospholipase A_2; PG, prostaglandin; TX, thromboxane; LPS, lipopolysaccharide; GSH, glutathione; ADAM, 9-anthryldiazomethane.

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Macrophages are known to produce PGE_2 via the COX-2-dependent pathway in response to proinflammatory stimuli such as interleukin-1, tumor necrosis factor, and lipopolysaccharide (LPS) over long-term culture (14, 15). In contrast, these cells produced TXB_2 in marked preference to PGE_2 when stimulated for several minutes with a Ca^{2+} ionophore (18). These observations led us to formulate a hypothesis that there is preferential, phase-specific correlation between the two COX isoforms and the different terminal PG synthases to provide particular prostanoids by a single cell population. We now provide evidence that LPS stimulation of macrophages is accompanied by induction of terminal PGE_2 synthase that functionally couples with COX-2, leading to increased capacity to produce PGE_2 in preference to other prostanoids.

MATERIALS AND METHODS

Materials. The commercially available materials and their sources were as follows: LPS (*E. coli* 0111:B4), bovine serum albumin, actinomycin D, and cycloheximide, from Sigma; soluble starch, from Wako; bacto-peptone, from Difco; arachidonic acid, from Nu-chek Prep., Inc.; 9-anthryldiazomethane (ADAM), PGE_2, PGD_2, and TXB_2, from Fnakoshi; and PGH_2 and cDNA probe for mouse COX-1, from Cayman Chemical. NS-398 (19) was a generous gift from Taisho Pharmaceutical Co. Ltd. Rabbit antisera to mouse COX-1 and -2 were donated by Dr. W.L. Smith (Michigan State University, East Lansing, MI).

Isolation and activation of rat peritoneal macrophages. Adherent macrophages were prepared from the peritoneal cells of male Sprague-Dawley rats (Japan SLC) 4 days after the injection of 5% soluble starch and 5% bacto-peptone in saline (5 ml/100 g body weight) as described previously (18). In short, peritoneal cells obtained from 3-4 rats were mixed and seeded in 35-mm diameter plastic dishes (Nunclon Delta) at a cell density of 1.5×10^6 cells/ml in 3 ml of RPMI 1640 medium (GIBCO BRL) containing 10% (v/v) fetal bovine serum (Bio Whittaker). After settlement for 2 h, almost all of the adherent cells were macrophages as assessed by microscopic examination of esterase activity and Giemsa staining. Cell viability throughout the experiments was around 95% by examination with trypan blue dye exclusion. In some experiments, the cells were pretreated with 100 μM aspirin to inactivate constitutively expressed COX during the 2-h settlement. Then the cells were washed, and incubated in the medium with or without 10 μg/ml LPS up to 12 h. In some experiments, the cells were cultured in the presence of 3.6 μM cycloheximide or 1 μM actinomycin D to inhibit new protein or RNA synthesis. All incubation procedures were performed with 5% CO_2 in humidified air at 37 °C.

Conversion of exogenous arachidonic acid to prostanoids. For estimation of the ability of the macrophages to yield COX metabolites from exogenous arachidonic acid, macrophages that had been cultured with or without LPS were washed, incubated for 15 min with Hank's balanced salt solution containing 0.1% bovine serum albumin, and then incubated for additional 40 min with 30 μM arachidonic acid. In some experiments, cells were incubated with 50 μM indomethacin, a non-selective COX inhibitor (20), or with 1 μM NS-398, a COX-2-selective inhibitor (19). Culture supernatants were harvested, and prostanoids were extracted with a Sep-Pak C18 cartridge (Millipore) and derivatized with ADAM to the fluorogenic products for application to high-performance liquid chromatography as described previously (18).

SDS–PAGE/immunoblotting. Cells were washed once with 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl [phosphate-buffered saline (PBS)] and lyzed in PBS containing 0.1% SDS at 1×10^7 cells/ml. The lysate was applied to SDS-polyacrylamide gels (TEFCO) and electrophoresed under reducing conditions. Separated proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schuell) by a semi-dry blotter (MilliBlot-SDE system; Millipore) according to the manufacturer's instruction. The membranes were then washed once with TBS, pH 7.2, containing 0.1% Tween-20 (TBS-T) and then blocked for 1 h in TBS-T containing 3% skim milk. Then the antibody against mouse COX-1 or COX-2 was added at a dilution of 1:3,000 in TBS-T and incubated for 2 h. Following three washes with TBS-T, the membrane was treated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed; 1:7,000 dilution) in TBS-T. After six washes, the protein bands were visualized with an ECL Western blot analysis system (Amersham).

Measurement of each prostanoid synthase activity. Macrophages, seeded at 1.5×10^6 cells/ml in 30 ml/150 $\times$ 25 mm plastic dish (Corning, NY), were incubated with or without 10 μg/ml LPS, washed several times with the medium, and then scraped off the dish. The cells were disrupted by sonication in 400 μl of 2 M Tris-HCl, pH 8.0, with a Branson Sonifier Model 250, and the supernatants were used as macrophage lysates after centrifugation of the sonicates at 1,700 $\times$ *g* for 10 min at 4 °C. For assessment of PGE_2 synthase activity (11, 12), an aliquot of each lysate (100 μg protein equivalents) was incubated with 2 μg of PGH_2 for 30 sec at 24 °C in a 0.1 ml of 1 M Tris-HCl, pH 8.0, containing 2 mM GSH. PGD_2 synthase activity (7-10) was measured by mixing an aliquot of the macrophage cell lysates (100 μg protein equivalents) with 2 μg of PGH_2 for 30 sec at 24 °C in a 0.1 ml of 1 M Tris-HCl, pH 8.0, containing 2 mM GSH and 1 mM *p*-chloromercuriphenylsulfonic acid. TX synthase activity (4-6) was measured by incubating an aliquot of the lysates (100 μg protein equivalents) with 2 μg of PGH_2 for 30 sec at 24 °C in a 0.1 ml of 1 M Tris-HCl, pH 7.4. Each reaction was terminated by the addition of 100 mM $FeCl_2$. For assessment of PGE_2 and PGD_2, the reaction mixtures were left at room temperature for 15 min, mixed with 13, 14-dihydro-15-dehydro-$PGF_{2\alpha}$ as an internal standard, adjusted to pH 3.0, further mixed vigorously with 3 ml of ether and 0.5 g of sodium sulfate, and centrifuged for 10 min at 430 $\times$ *g* at 4 °C. Extraction was repeated twice, and the pooled ether phase was condensed by evaporation. The resulting residue was reacted with ADAM and analyzed by HPLC as described above. TXB_2 was quantified by use of an enzyme immunoassay kit (Cayman Chemical).

Statistical analysis. Data were expressed as the mean $\pm$ SEM of more than three independent experiments. Statistical analysis was employed with Student's *t*-test.

RESULTS

Conversion of exogenous arachidonic acid to different prostanoids. The ability of macrophages to produce various prostanoids was assessed by following the metabolism of exogenous arachidonic acid to the final products (18). When unstimulated cells were incubated with 30 μM exogenous arachidonic acid, they produced TXB_2 most abundantly, reaching 35.9 $\pm$ 2.7 ng/dish, followed by PGD_2, reaching 9.0 $\pm$ 1.9 ng/dish, and only a small amount of PGE_2, reaching 1.9 $\pm$ 0.6 ng/dish (n = 8) (Fig. 1A). The conversion of exogenous arachidonic acid to PGE_2 increased after stimulation with LPS, reaching a maximal 120.8 $\pm$ 15.9 ng/dish (n = 8) after 12 h of culture. In contrast, the conversion to PGD_2 and TXB_2 did not change appreciably during the overall culture periods. Thus, the ability of macrophages to metabolize exogenous arachidonic acid to PGE_2 in-

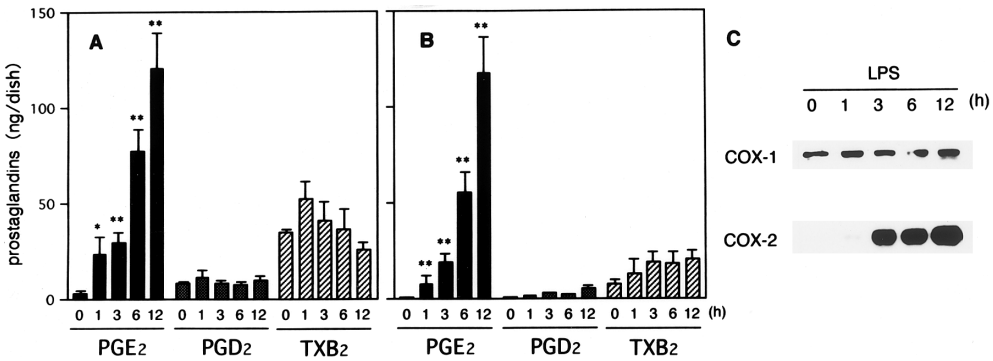

FIG. 1. Changes in the ability of macrophages to produce prostanoids from exogenous arachidonic acid during incubation with LPS and the effect of aspirin pretreatment. Peritoneal macrophages (4.5×10^6 cells/3 ml/dish) were allowed to settle in a dish for 2 h in the absence (A) or presence (B) of 100 μM aspirin, and after extensive washings they were cultured for up to 12 h with 10 μg/ml LPS. Then the cells were washed with the medium at the indicated time, and 30 μM exogenous arachidonic acid was added. After 40 min, the supernatants were harvested, and prostanoids were extracted and quantified as described in Materials and Methods. Amounts of produced PGE_2, PGD_2, and TXB_2 are expressed as ng/dish. Values are expressed as the mean ± SE of eight independent experiments. * and ** indicate a statistically significant difference from the corresponding control value at $p < 0.01$ and $p < 0.05$, respectively. The cells derived from (A) were harvested at each time point and subjected to immunoblotting with antibodies against COX-1 and COX-2 (C).

creased selectively about 50-fold by 12 h after LPS stimulation, in comparison to TXB_2 and PGD_2 generation that was unchanged.

The increase in PGE_2 formation paralleled the induction of COX-2 protein expression (Fig. 1C). COX-2, which was minimally expressed before culture, increased significantly from 3 to 12 h to reach a peak in cells that were cultured with LPS. Constitutive expression of COX-1 did not change appreciably during the same periods of culture.

Pharmacological studies. Inactivation of the constitutive COX by pretreatment of the cells with aspirin abolished the generation of all prostanoids by unstimulated cells and reduced PGD_2 and TXB_2 formation markedly during the overall culture periods (Fig. 1B). On the other hand, the conversion to PGE_2 by cells cultured for 3 to 12 h with LPS was not affected appreciably by aspirin pretreatment (Fig. 1B), indicating that increased PGE_2 generation largely depended upon the inducible COX. Irrespective of the nearly 50-fold increase in PGE_2 formation during the culture of aspirin-pretreated cells with LPS, PGD_2 increased only modestly over the control even after 12 h of culture with LPS, at which time COX-2 was abundantly expressed. LPS stimulation increased TXB_2 formation by aspirin-pretreated cells, which reached about a 3-fold increase over the control at 3-12 h.

When unstimulated cells were incubated with arachidonic acid in the presence of 1 μM NS-398, a COX-2 inhibitor, the conversion of exogenous arachidonic acid to neither PGE_2, PGD_2 nor TXB_2 was suppressed significantly (Table 1). In contrast, NS-398 inhibited the conversion to PGE_2 by the cells cultured for 12 h with LPS by ~90%. PGD_2 generation by LPS-stimulated cells was not suppressed by NS-398 significantly, while TXB_2 formation by LPS-stimulated cells was inhibited partially by it. The addition of indomethacin, instead of NS-398, completely suppressed PGE_2, PGD_2 and TXB_2 formation by unstimulated and LPS-stimulated cells (Table 1). When cycloheximide or actinomycin D was added to culture to inhibit new protein or RNA synthesis, respectively, the conversion of arachidonic acid to PGE_2 by cells stimulated for 12 h with LPS was abolished almost completely (Table 1). Almost identical results were obtained when aspirin-pretreated cells were cultured for 12 h with LPS in the presence of cycloheximide or actinomycin D (data not shown). PGD_2 generation was not affected, and TXB_2 formation was suppressed only partially, by these agents (Table 1).

Changes in terminal prostanoid synthase activities. Selective increase in the ability to produce PGE_2 relative to other prostanoids following LPS stimulation suggests that PGE_2 synthase, the terminal enzyme in the PGE_2 biosynthetic pathway, is also inducible. To clarify this hypothesis, we examined the terminal prostanoid synthase activities in cell lysates by assessing the conversion of exogenous PGH_2 to each prostanoid (Fig. 2). Although PGE_2 synthase activity was undetectable in unstimulated cells, it increased dramatically after culture for 12 h with LPS. Increase in PGE_2 synthase activity by LPS was inhibited almost completely when cells were cultured in the presence of actinomycin D or cycloheximide, implying that the *de novo* induction of PGE_2 synthase accompanies the LPS-induced PGE_2 generation by these cells. PGD_2 synthase activity was unchanged during the entire culture period and was not affected by actinomycin D or cycloheximide. TX synthase activity in cells stimulated for 12 h with LPS was comparable to that in unstimulated

TABLE 1

Effect of COX Inhibitors (A) and Cycloheximide and Actinomycin D (B) on the Production of Prostanoids by Macrophages

Treatments	Culture periods (h)	PGE_2	PGD_2	TXB_2
A				
Control	0	2.0 ± 0.3	9.6 ± 0.3	35.9 ± 2.7
Indomethacin	0	ND	3.5 ± 1.2††	0.3 ± 0.2††
NS-398	0	2.9 ± 0.8	19.0 ± 3.2	40.2 ± 7.1
LPS	12	149.4 ± 25.1†	11.9 ± 2.0	30.3 ± 5.0
LPS + Indomethacin	12	3.2 ± 2.0**	0.9 ± 0.5**	2.3 ± 1.4**
LPS + NS-398	12	26.6 ± 6.2**	6.4 ± 1.1	8.1 ± 0.9*
B				
Control	0	1.9 ± 0.6	9.0 ± 1.9	35.9 ± 2.7
LPS	12	127.3 ± 16.5††	14.1 ± 3.7	31.4 ± 2.2
LPS + Cycloheximide	12	6.5 ± 3.6*	10.6 ± 3.3	12.0 ± 1.1**
LPS + Actinomycin D	12	5.8 ± 3.0 *	15.3 ± 2.4	6.7 ± 1.3**

Values are expressed as the mean ± SE of four independent experiments. †$p < 0.01$ and ††$p < 0.05$ *versus* control; and *$p < 0.01$ and **$p < 0.05$ *versus* LPS of each group. ND, not detected.

cells but was suppressed partially by cycloheximide or actinomycin D, suggesting the rapid turnover of this enzyme.

DISCUSSION

Recent studies have revealed that the two COX isoforms function independently even when both are expressed in the same cell, even though they catalyze the same enzyme reaction *in vitro* (13-15). The findings that macrophages and fibroblasts utilize only COX-2 for PGE_2 biosynthesis in response to LPS, growth factors, and mitogens, irrespective of the constitutive expression of COX-1 (14, 15), imply the particular PGE_2 biosynthetic route in which COX-2 and downstream PGE_2 synthase are functionally linked. In support for this, Harada *et al.* (20) reported that although both PGE_2 and TXB_2 levels increased in the exudate of rat carrageenin-induced pleurisy, only PGE_2 was markedly decreased by the treatment of rats with several COX-2-selective inhibitors. We previously showed that the PGE_2 level was elevated significantly in the pleural exudate of rats 3 h after injection of carrageenin (21), whereas at 1 h TXB_2 and 6-keto-$PGF_{1\alpha}$ were the major arachidonate metabolites compared with PGE_2 (22). Our present *in vitro* study using a single cell population, rat peritoneal macrophages, has revealed that the *de novo* induced COX-2 is linked to PGE_2 synthase that is also inducible, thereby causing the change in the capacity of the cells to produce different PG species before and after cell activation.

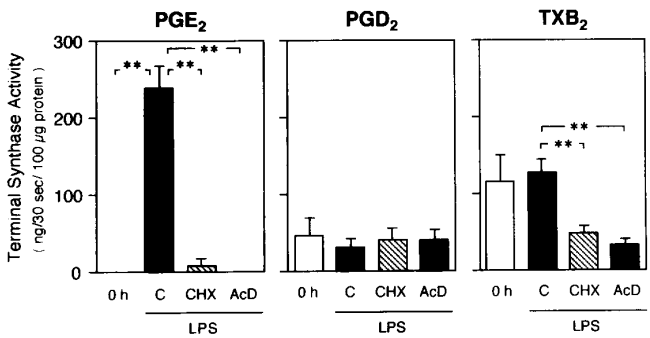

FIG. 2. Changes in terminal prostanoid synthase activities in the macrophage lysates. Peritoneal macrophages were stimulated with 10 μg/ml LPS in the presence of cycloheximide (CHX, 3.6 μM), actinomycin D (AcD, 1 μM), or vehicle (C) for 12 h. Enzymatic activities of PGE_2 synthase (*left*), PGD_2 synthase (*center*), and TXB_2 synthase (*right*) were determined as described in Materials and Methods. Values are expressed as the mean ± SE of three independent experiments. * and ** indicate a statistically significant difference from the corresponding control value at $p < 0.01$ and $p < 0.05$, respectively.

The profiles of PG produced from exogenous arachidonic acid by unstimulated macrophages differed from those produced by LPS-stimulated cells: TXB_2, followed by PGD_2, was the major metabolite produced by unstimulated macrophages with minimal formation of PGE_2, whereas replicate cells that had been cultured for 12 h with LPS produced more PGE_2 than TXB_2 and PGD_2. Thus, the ability to generate PGE_2 was induced rather specifically in LPS-stimulated macrophages. The induction of PGE_2 generation correlated with the incremental expression of COX-2 protein. Several pharmacological studies confirmed the close correlation between COX-2 and PGE_2 biosynthesis, which has been also demonstrated by other convincing studies (13-15). Measurement of terminal prostanoid synthase activities revealed that PGE_2 synthase was found to be an inducible enzyme. The increase in PGE_2 synthase activity was completely suppressed by protein and RNA synthesis inhibitors, implying the stimulus-initiated

induction of this particular terminal prostanoid synthase, rather than posttranslational activation of the pre-existing enzyme. Thus, the high inducibility of this terminal synthase appears to be one of the most important factors for the differential production of PGs at different stages of cell activation.

Our results also indicate that TXB_2 and PGD_2 generation is coupled to the constitutive COX-1 in unstimulated macrophages. The functional correlation between COX-1 and TX and PGD_2 synthases is compatible with their coupling for rapid TXA_2 production by activated platelets (23) and for IgE-dependent immediate PGD_2 generation by activated mast cells (10, 13), respectively. The approximately 3-fold increase in TXB_2 formation by aspirin-pretreated, LPS-treated cells during culture with LPS indicate that COX-2 can also supply PGH_2 to TX synthase, albeit to a much lesser extent than to PGE_2 synthase. That both COX-1 and COX-2 couple to TX synthase has been recently shown in endothelial cells (24). In contrast, PGD_2 generation appears to be functionally linked to COX-1 more tightly than COX-2 in macrophages, since the conversion of exogenous arachidonic acid to PGD_2 was unchanged over the culture period, was abolished by specific inhibition of pre-existing COX-1, and was insensitive to NS-398, even when COX-2 expression and PGE_2 generation increased greatly. Conversely, the fact that PGE_2 generation by LPS-stimulated cells was largely dependent upon COX-2, even though COX-1, with which TX and PGD_2 synthases are functionally coupled, also exists in the same cell, suggests that the newly induced PGE_2 synthase is functinally linked to COX-2 in preference to COX-1. Thus, the two COX isoforms may couple to distinct terminal PG synthases preferentially, and this may be one of the explanation why the two COX isoforms are functionally segregated even when both isoforms are expressed in the same cell.

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