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### Research Article

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# ***sn*-1,2-Diacylglycerols and Phorbol Diesters Stimulate Thromboxane Synthesis by *De Novo* Synthesis of Prostaglandin H Synthase in Human Promyelocytic Leukemia Cells**

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## **Abstract**

We studied the regulation of thromboxane (TX) synthesis in promyelocytic leukemia cells during macrophage differentiation. Cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) showed rates of TXB<sub>2</sub> synthesis from exogenous arachidonic acid that exceeded that of control cells by a factor of up to 81. Cells treated with *sn*-1,2-dioctanoylglycerol (diC8) showed similarly high TXB<sub>2</sub> synthesis rates when diC8 was added concomitantly with a subthreshold concentration of TPA or when given in multiple doses. These activities depended on *de novo* synthesis of prostaglandin H (PGH) synthase because: (a) microsomal PGH synthase activity showed large increases in  $V_{max}$  values, and (b) mass measurements of PGH synthase revealed the presence of PGH synthase in differentiating cells whereas the enzyme was undetectable in control cells. These results indicate that macrophage differentiation is associated with stimulation of TXB<sub>2</sub> synthesis that requires both activation of protein kinase C and *de novo* synthesis of PGH synthase.

## **Introduction**

Infiltration of monocytes into affected tissues occurs in diseases as varied as rheumatoid arthritis (1), chronic inflammatory bowel disease (2), hydronephrosis (3–5), and atherosclerosis (6), whereupon the monocytes undergo extensive morphologic and functional alterations that resemble those observed after induction of macrophage differentiation *in vitro* (7–9). Thus monocyte infiltration and differentiation are clearly key events in the inflammatory process.

To understand these events better, it will be necessary to establish the sequence of reactions that underlies monocyte/macrophage differentiation and to identify the control mechanisms that are involved. One such mechanism may depend on

the synthesis of thromboxane (TX).<sup>1</sup> Monocytes/macrophages, maintained *in vitro*, release TX along with other biologically active molecules (3–5, 10). Moreover, *in vivo* models of inflammation, such as the hydronephrotic kidney (5) and myocardial infarction (11, 12), have provided evidence that TX synthesized by monocytes/macrophages is important in modulating the inflammatory reaction at sites of tissue injury.

The objective of the present investigation was to obtain information about the regulation of TX synthesis in differentiating monocytes. We used differentiating HL-60 cells as a model system. Several studies (7, 8, 13–15) have shown that these cells differentiate into macrophage-like cells in response to phorbol diesters, and evidence has indicated a close relation between the action of these esters and the activity of protein kinase C (PKC) (16–18). Furthermore, Ebeling et al. (13) added the cell-permeable diacylglycerol, dioctanoylglycerol (diC8), to HL-60 cells and showed that it was able to stimulate both PKC and cell adhesion. The same investigators showed that HL-60 macrophage differentiation only occurred after repeated addition of diC8 and that repeated addition of diC8 induced persistent occupancy of the phorbol diester binding site. In contrast, a single addition of diC8 led to transient occupancy of the phorbol diester binding site and did not induce macrophage differentiation, and a recent report by May et al. (15) showed that diC8 was rapidly phosphorylated to form phosphatidic acid in HL-60 cells. These results, in particular, strongly suggested that PKC activation—if maintained for several hours—is a key event in macrophage differentiation.

We therefore used either 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or diC8 to initiate macrophage differentiation, and asked the following questions: (a) When during the course of monocyte/macrophage differentiation is the prostaglandin H (PGH) synthase pathway activated? (b) Does the regulation of this pathway involve PKC? and (c) does it involve formation of PGH synthase? Our results provide new information regarding the control of TXB<sub>2</sub> synthesis during the early stages of macrophage differentiation *in vitro*.

## **Methods**

**Materials.** Cell culture media were obtained from Gibco, Grand Island, NY; standard PGs were obtained from Seragen, Boston, MA; [<sup>3</sup>H]TXB<sub>2</sub> (sp act 139 Ci/mmol), [<sup>3</sup>H]PGE<sub>2</sub> (sp act 120 Ci/mmol), [<sup>3</sup>H]6-keto-PGF<sub>1 $\alpha$</sub>

1. **Abbreviations used in this paper:** AA, arachidonic acid; ALP, alkyl-lysophospholipid; diC8, *sn*-1,2-dioctanoylglycerol; LDH, lactate dehydrogenase; PDS, plasma-derived serum; PKC, protein kinase C; TFP, trifluoperazine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TX, thromboxane.

Preliminary results of this study were presented at the Sixth International Conference on Prostaglandins and Related Compounds, Florence, Italy, 3–6 June 1986.

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(sp act 120 Ci/mmol) were obtained from New England Nuclear, Boston, MA; TPA, arachidonic acid (AA), and Protein A-Sepharose CL-4B were obtained from Pharmacia, Freiburg, Federal Republic of Germany; antibodies against icosanoids were obtained from the Institut Pasteur, Paris, France; monoclonal antibodies against sheep seminal vesicle PGH synthase (cyo-1, cyo-5) and partially purified sheep seminal vesicle PGH synthase were from Oxford Biomedical Research, Inc., Oxford, MI; H-7 from Seikagaku America Inc., St. Petersburg, FL;  $\text{Ca}^{2+}$  ionophore A23187, cycloheximide, and all other reagents were from Sigma Chemical Co., Munich, Federal Republic of Germany; diC8 at 3 M and TPA at 10 mM in acetone was stored under argon at  $-80^{\circ}\text{C}$ . Double-distilled  $\text{H}_2\text{O}$  was used throughout.

**Cell culture.** The human promyelocytic leukemia cell line HL-60 was obtained from the American Type Culture Collection, Rockville, MD, and maintained in RPMI 1640 medium containing 10% fetal calf serum, 100 U/liter penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Experiments designed to determine levels of  $\text{TXB}_2$  were performed in 5% calf plasma-derived serum (PDS) (19, 20) because  $\text{TXB}_2$  in PDS was not detectable whereas that in 5% fetal calf serum ranged between 300 and 800  $\text{pg}/\text{ml}$ . The diC8 and  $\text{Ca}^{2+}$  ionophore A23187 were added to cells in concentrated solutions of 2–5  $\mu\text{l}$  acetone or 6  $\mu\text{l}$  ethanol, respectively, to give the concentrations indicated in the figure legends. Control cells received acetone or ethanol instead. Cell viability tests were performed under all experimental conditions by both trypan blue exclusion and release of lactate dehydrogenase (LDH): > 95% of the cells excluded trypan blue and significant release of LDH was obtained only at concentrations of TPA beyond 200 nM or concentrations of diC8 beyond 150  $\mu\text{M}$ .

**Radioimmunoassay (RIA).** The concentration of icosanoids was determined by RIA according to Granstrom and Kindahl (21) after reversed-phase extraction and thin-layer chromatography as described previously for  $\text{PGE}_2$  and 6-keto-PGF $_{1\alpha}$  (19, 20). Total recovery for  $\text{TXB}_2$  ranged between 44% and 67%, cross-reactivity at 50% binding was < 0.2% for  $\text{PGA}_2$ ,  $\text{PGB}_2$ ,  $\text{PGE}_2$ ,  $\text{PGD}_2$ ,  $\text{PGF}_{2\alpha}$ , 6-keto-PGF $_{1\alpha}$ , AA, and  $\text{PGH}_2$ . Water samples subjected to exactly the same analytical procedure as biological samples ranged between 1 and 4  $\text{pg}/\text{ml}$ , intraassay variation was 6%–11%, and interassay variation was 9%–14%.

**Estimation of HL-60 PGH synthase mass.** The amount of PGH synthase protein in HL-60 cells was estimated by using two monoclonal antibodies directed against different antigenic sites on sheep seminal vesicle PGH synthase (cyo-1, cyo-5) (22, 23). Solubilized microsomes were prepared as described under the subsection "Assay of microsomal PGH synthase activity" except that the microsomes were taken up in assay buffer (50 mM Tris, pH 7.8, 2 mM EDTA, 0.25 M sucrose, 0.15% Tween 20). Purification of cyo-1 and cyo-5 antibodies: 15 ml of culture medium containing the cyo-1 and cyo-5 antibodies, respectively, was purified by Protein A-Sepharose column chromatography as described by Smith and Rollins (22). Purified cyo-1 antibody was iodinated with  $^{125}\text{I}$  using chloramine T (24) (sp act 20,350 dpm/ng of protein). 50  $\mu\text{l}$  of 125 mM borate buffer, pH 8.4, containing 2  $\mu\text{g}$  of purified unlabeled cyo-5 antibody was incubated in microtiter plates (Flow General Inc., McLean, VA) for 18 h at  $4^{\circ}\text{C}$ . Solubilized HL-60 microsomes or partially purified sheep seminal vesicle PGH synthase dissolved in 50  $\mu\text{l}$  of assay buffer was added and incubated for 75 min at room temperature; the plates were incubated with buffer (1% Tween 20, 5% bovine serum albumin dissolved in phosphate-buffered saline, pH 7.4) for 10 min at room temperature. 50  $\mu\text{l}$  of buffer containing purified  $^{125}\text{I}$ -iodinated cyo-1 antibody (53,000 dpm) was added and incubated for 50 min at room temperature. Bound radioactivity was counted in a LKB Wallac 1280 Ultragamma scintillation spectrometer (LKB Produkter, Bromma, Sweden). Background samples that did not contain unlabeled antibody (cyo-5), solubilized HL-60 microsomes, or standard PGH synthase ranged between 15%–20% of the biological samples. Standard curves were generated using PGH synthase partially purified from sheep seminal vesicle microsomes. The results obtained with HL-60 cells are reported in picograms of partially purified sheep seminal vesicle PGH synthase per microgram of HL-60 microsomal protein.

**Assay of microsomal PGH synthase activity.** PGH synthase activity was determined according to Sheng et al. (4) and Whiteley and Ne-

dleman (26) with minor modifications. Briefly, HL-60 cells were cultured as described under cell culture. HL-60 cells at a density of  $1.0 \times 10^6/\text{ml}$  were suspended in culture medium supplemented with 5% PDS. Experimental solutions were added as described under cell culture and the cells were incubated for 12 h. Then cells were washed twice with homogenization buffer (50 mM Tris-HCl, 2 mM EGTA, 0.3 mM  $\text{MgCl}_2$ , 1% free fatty acid-free albumin, 25% sucrose, pH 7.8), and cells at a density of  $10^7/\text{ml}$  were homogenized using a Tekmar homogenizer equipped with an 18-cm shaft (Janke und Kunkel, Staufen, Federal Republic of Germany) for  $12 \times 10$  s on ice in siliconized glass tubes under argon. The homogenate was centrifuged at 1,000  $g$  for 5 min at  $4^{\circ}\text{C}$  to remove cell debris, nuclei, and mitochondria. The supernatant was transferred under argon to a polycarbonate tube and centrifuged at 55,000  $g$  for 15 min. The resulting pellet was washed three times under argon on ice using 2 ml of 50 mM Tris buffer, pH 7.8, 2 mM EGTA, 25% sucrose. Protein was determined by the method of Lowry et al. (27). The reaction was started by addition of 20  $\mu\text{g}$  of microsomal protein that was suspended in 20  $\mu\text{l}$  of homogenization buffer to 480  $\mu\text{l}$  of assay buffer (50 mM Tris HCl, 2 mM EGTA, 1 mM reduced glutathione, 1.2 mM epinephrine) at  $37^{\circ}\text{C}$  for 7.5 min. Before addition of microsomes, AA was added in a concentrated solution of 5  $\mu\text{l}$  of ethanol. The reaction was stopped by addition of 2.5 ml of ice-cold PBS acidified to pH 3.4 and immediately extracted using Octadecyl silica gel columns as described (19). At concentrations of AA between 4 and 128  $\mu\text{M}$ , the formation of  $\text{TXB}_2$ ,  $\text{PGE}_2$ , and 6-keto-PGF $_{1\alpha}$  was linear for 12 min. Formation of icosanoids was also linear with respect to microsomal protein (determined between 5 and 50  $\mu\text{g}$ ). Control microsomes boiled for 10 min or preincubated with 100  $\mu\text{M}$  aspirin for 25 min on ice did not contain measurable PGH synthase activity. Concentrations of icosanoids were determined by RIA as described above.

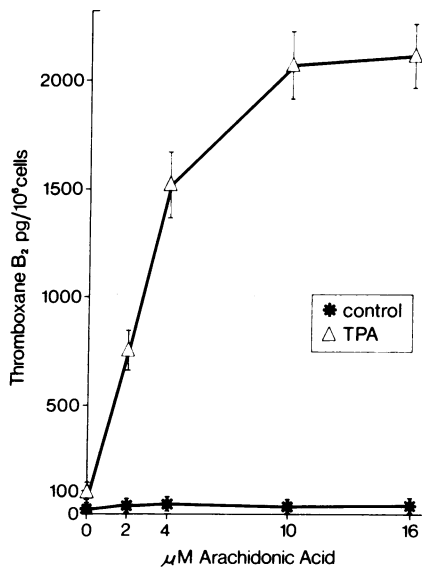
## Results

**TPA-dependent stimulation of  $\text{TXB}_2$  synthesis from exogenous AA.** Preliminary experiments, performed with HL-60 cells that were incubated in the absence of unesterified AA, revealed that the TPA-induced differentiation of these cells into macrophages is accompanied by increased  $\text{TXB}_2$  synthesis. Unstimulated cells synthesized 20–105  $\text{pg}$  of  $\text{TXB}_2/10^6$  cells per 12 h, whereas cells that were stimulated with 10 nM TPA synthesized from 3.7 to 9.8 times this amount (results not shown). These results extended to HL-60 cells the report of Ohuchi et al. (28) and others that phorbol diesters stimulate the synthesis of PGs in cells in culture.

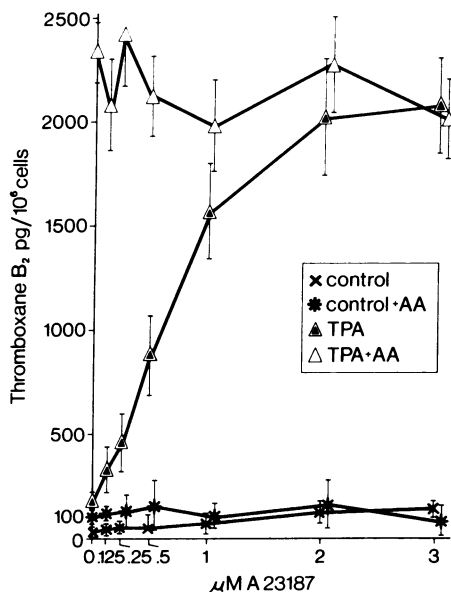
To determine whether the action of TPA on HL-60 cells was associated with increased activity of enzymes in the PG synthesis pathway, we tested the effect of added unesterified AA on the synthesis of  $\text{TXB}_2$  in control and TPA-treated cells (Fig. 1). We found that AA had little effect on  $\text{TXB}_2$  synthesis in undifferentiated control cells, but increased  $\text{TXB}_2$  synthesis by as much as 81-fold in TPA-treated cells at a concentration of 10  $\mu\text{M}$ .

Interestingly, the effect of the  $\text{Ca}^{2+}$  ionophore A23187 (29) on HL-60 cells resembled that of added AA. Undifferentiated cells that were treated with the ionophore produced little  $\text{TXB}_2$  in the presence or absence of AA. In contrast, TPA-treated cells that were incubated in the presence of 1–3  $\mu\text{M}$  ionophore produced maximal amounts of  $\text{TXB}_2$  even in the absence of added AA (Fig. 2). Thus the ionophore seemed unable by itself to increase the activity of enzymes in the PG synthesis pathway. It was, however, able to stimulate  $\text{TXB}_2$  synthesis, presumably by promoting the release of AA from endogenous phospholipid stores.

To determine whether the effect of TPA on  $\text{TXB}_2$  synthesis occurred early during the course of macrophage differentiation,



**Figure 1.** Concentration dependence of TXB<sub>2</sub> synthesis from exogenous AA in undifferentiated and TPA-treated HL-60 cells. Cells at a density of  $1 \times 10^6$ /ml were cultured in 60-mm Falcon plastic dishes in 5 ml of RPMI 1640 medium supplemented with 5% PDS. TPA dissolved in acetone was added to medium to give a final concentration of TPA of 10 nM and 0.01% acetone or acetone as control. 11 h later parallel cultures were incubated with increasing concentrations of AA dissolved in a concentrated solution of 10  $\mu$ l ethanol to give a final concentration of ethanol of 0.3% or ethanol as control. After 60 min the concentration of TXB<sub>2</sub> was determined by RIA as described in Methods. The data points represent the means of three parallel dishes  $\pm$  standard deviation.



**Figure 2.** Effects of Ca<sup>2+</sup> ionophore A23187 on TXB<sub>2</sub> synthesis in control and TPA-treated HL-60 cells. Cells were cultured and TPA added as described in Fig. 1. 11 h later parallel cultures were incubated with increasing concentrations of Ca<sup>2+</sup> ionophore A23187 dissolved in a concentrated solution of 10  $\mu$ l of ethanol or ethanol in the presence of 10  $\mu$ M AA as described in Fig. 1 and in Methods. 60 min later the concentration of TXB<sub>2</sub> was determined as described in Methods. The data points represent means of duplicate determinations  $\pm$  standard deviation.

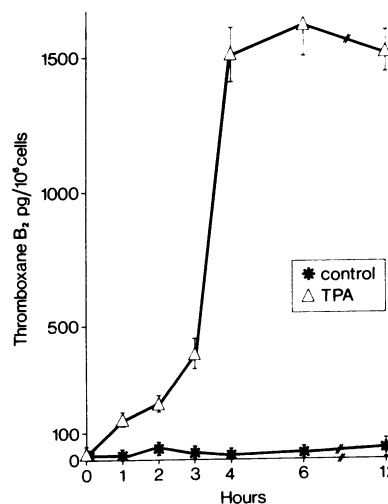
we incubated HL-60 cells with TPA and measured the rate of TXB<sub>2</sub> synthesis in the presence of added AA as a function of time (Fig. 3). We observed stimulatory effects of TPA within 1 h, which increased sharply after 3 h and plateaued after 4–6 h. By comparison, TPA-induced adherence of the cells to the culture dish began after 12 h, and  $\sim$  95% of the cells adhered to the dish and showed macrophage morphology (7) within 24 h.

We next determined whether TPA stimulated the formation of other products of the PGH synthase pathway and found (Table I) that all major products of this pathway, TXB<sub>2</sub>, PGE<sub>2</sub>, and 6-keto-PGF<sub>1 $\alpha$</sub> , increased in response to the phorbol diester. This raised the possibility that TPA may have promoted the activation of at least one enzyme involved in PG synthesis.

To determine whether the effect was specific for phorbol diesters that activate PKC, we used the phorbol diester, 4- $\alpha$ -phorbol 12,13-didecanoate. This phorbol diester is unable to activate PKC in several biological systems (30). We found that it was also unable to stimulate TXB<sub>2</sub> synthesis and induce cell adherence in HL-60 cells, even when its concentration exceeded the maximal concentration of TPA by two orders of magnitude (not shown).

**Diacylglycerol-dependent synthesis of TXB<sub>2</sub>.** Experiments with the cell-permeable diacylglycerol, diC8 (15, 16), provided further evidence for a relation between PKC activation, TXB<sub>2</sub> synthesis, and macrophage differentiation. We added diC8 to undifferentiated cells and determined the ability of the cells to produce TXB<sub>2</sub> in the presence of added AA. 90  $\mu$ M diC8 significantly stimulated TXB<sub>2</sub> synthesis (Fig. 4 A), reaching 27% of the maximal TPA effect (Fig. 4 B). Moreover, when a sub-threshold concentration of TPA (0.1 nM) was included in the culture medium, concentrations of diC8 between 10 and 30  $\mu$ M induced maximal synthesis of TXB<sub>2</sub> (Fig. 4 A). Other species of diacylglycerol, such as didecanoate and 1-oleoyl-2-acetyl diacylglycerol, also showed synergistic effects in the presence of sub-threshold concentrations of TPA although they were not as active as diC8 (results not shown).

We next determined the kinetics of diC8-dependent TXB<sub>2</sub> synthesis in the presence of 0.1 nM TPA (Fig. 5). We found that diC8 significantly stimulated the synthesis of TXB<sub>2</sub> (Fig. 5), PGE<sub>2</sub>, and 6-keto-PGF<sub>1 $\alpha$</sub>  (results not shown) within 1 h and its effect was maximal within 4–6 h. Because Ebeling et al. (13) had previously shown that diC8 is relatively inactive when added at



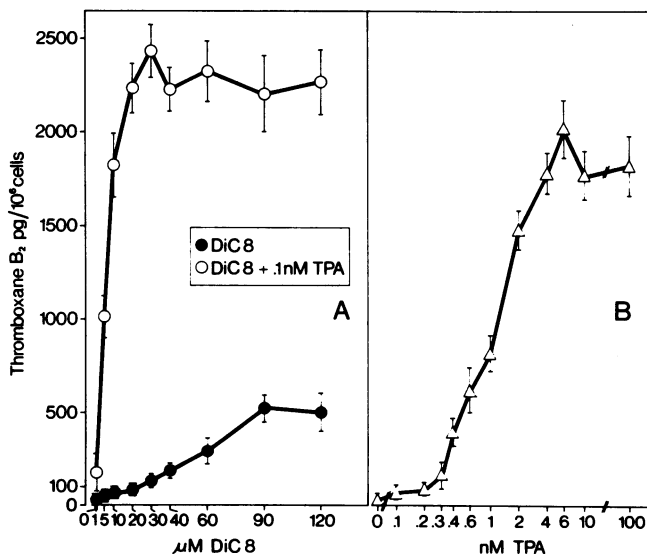
**Figure 3.** Kinetics of TPA-dependent TXB<sub>2</sub> synthesis from exogenous AA. Cells were cultured and TPA added as described in Fig. 1. 60 min before the time points indicated in the Figure, AA at 10  $\mu$ M was added to the cultures and the concentration of TXB<sub>2</sub> was determined as described in Methods. The data points represent means of duplicate dishes  $\pm$  standard deviation.

**Table 1. TPA-induced Macrophage Differentiation Is Associated with Enhanced Synthesis of the Major Products of the PGH Synthase Pathway from Exogenous AA**

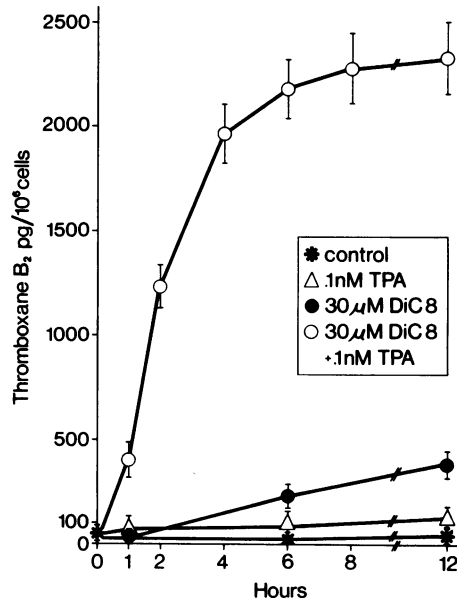
Icosanoid	Control	TPA
	pg/10 <sup>6</sup> cells	pg/10 <sup>6</sup> cells
TXB <sub>2</sub>	38±19	1,432±68
PGE <sub>2</sub>	105±14	987±59
6-keto-PGF <sub>1α</sub>	52±26	252±35

Cells were cultured and TPA was added as described in Fig. 1. 11 h later, control and TPA-stimulated cells were incubated for an additional 60 min in the presence of 10 μM AA and the concentration of icosanoids was determined as described in Methods. Numbers represent means of triplicate cultures±standard deviation.

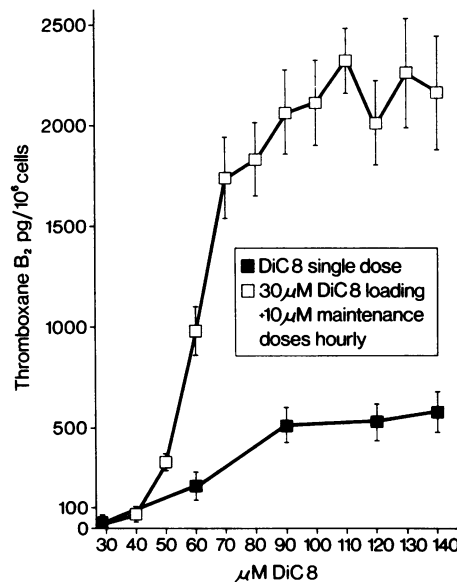
a single dose, but induces macrophage differentiation when added repeatedly at hourly intervals, we used a similar approach to determine whether maximal effects of diC8 on TXB<sub>2</sub> synthesis could be achieved in the absence of TPA. We added diC8 at an initial loading dose of 30 μM and in hourly maintenance doses of 10 μM, then measured the synthesis of TXB<sub>2</sub> from exogenous AA at a fixed time interval, 11–12 h after addition of the initial dose of diC8. As shown in Fig. 6 diC8 maximally stimulated TXB<sub>2</sub> synthesis when this dosage regimen was maintained for at least 5 h. Furthermore, diC8-dependent synthesis correlated closely with diC8-dependent cell adhesion. When a single low dose (30 μM) or high doses (90–140 μM) of diC8 was added to the cells, no more than 2%–5% of the cells attached to the dish within 48 h. In contrast, when the cells were given a loading



**Figure 4.** The diC8 and TPA synergistically stimulate TXB<sub>2</sub> synthesis. Cells were cultured as described in Fig. 1. (A) TPA and diC8 were added in concentrated solutions of acetone to give a concentration of 0.07% of acetone or acetone. (B) TPA was added in a concentrated solution to give a final concentration of acetone of 0.01% or acetone. After 11 h parallel cultures were incubated with 10 μM AA for 60 min and the concentration of TXB<sub>2</sub> was determined as described in Methods. The data points represent means of duplicate dishes±standard deviation.



**Figure 5.** Kinetics of diC8-dependent TXB<sub>2</sub> synthesis in the presence of 0.1 nM TPA. Cells were cultured and 30 μM diC8 and 0.1 nM TPA added as described in Fig. 4. 60 min before the time points indicated in the figure, parallel cultures were incubated with 10 μM AA and the concentration of TXB<sub>2</sub> was determined as described in Methods. The data points represent means of duplicate dishes±standard deviation.



**Figure 6.** When given at multiple doses diC8 induces maximal TXB<sub>2</sub> synthesis. Cells were cultured as described in Fig. 1 and a loading dose of 30 μM diC8 dissolved in a concentrated solution of 3 μl of acetone or acetone as control was added. Parallel cultures received additional maintenance doses at 10 μM diC8 dissolved in a concentrated solution of 3 μl acetone or acetone as control at hourly intervals for increasing time periods. The cultures were incubated with 10 μM AA at 11 h for 60 min and the concentration of TXB<sub>2</sub> was determined as described in Methods. 10 nM TPA induced synthesis of 2,160±193 pg TXB<sub>2</sub>/10<sup>6</sup> cells, and 140 μM diC8 added at a single dose induced 634±68 pg TXB<sub>2</sub>/10<sup>6</sup> cells in this experiment. Repeated addition of diC8 or acetone did not compromise viability as determined by trypan blue exclusion. The data points represent means of duplicate dishes±standard deviation.

dose of 30  $\mu\text{M}$  diC8 followed by four hourly maintenance doses of 10  $\mu\text{M}$  diC8, > 95% of the cells attached within 24 h.

Trifluoperazine (TFP) has previously been shown to inhibit the activity of PKC in HL-60 cells and the activity of PKC purified from bovine heart (31–33). We therefore determined whether TFP was able to inhibit the effect of TPA on TXB<sub>2</sub> synthesis. We found that TFP did indeed inhibit this effect (Table II), and that the concentrations of TFP required were similar to those reported to inhibit PKC-dependent protein phosphorylation in HL-60 cells (32). Furthermore, there was a close correlation between the concentrations of TFP required to inhibit TXB<sub>2</sub> synthesis and those required to inhibit HL-60 macrophage differentiation (results not shown). The alkyllysophospholipid, ET-18-OCH<sub>3</sub> (ALP), and the lipoidal amine, CP-46,665-1 (CP), have been shown to inhibit PKC in HL-60 cells (34, 35) by competing with the phospholipid activator of PKC phosphatidylserine.

Table II. Inhibitors of PKC Prevent Both TPA-induced Macrophage Differentiation and Synthesis of TXB<sub>2</sub>

Additions	TXB <sub>2</sub> pg/10 <sup>6</sup> cells
<b>Experiment I</b>	
Control	24±6
10 nM TPA	1,964±206
10 nM TPA + 1 $\mu\text{M}$ TFP	1,320±134
10 nM TPA + 5 $\mu\text{M}$ TFP	840±128
10 nM TPA + 50 $\mu\text{M}$ TFP	547±105
<b>Experiment II</b>	
Control	84±21
10 nM TPA	2,214±320
10 nM TPA + 1 $\mu\text{M}$ ALP	1,610±135
10 nM TPA + 5 $\mu\text{M}$ ALP	716±205
10 nM TPA + 500 nM CP	1,688±267
10 nM TPA + 1 $\mu\text{M}$ CP	1,240±184
10 nM TPA + 5 $\mu\text{M}$ CP	589±183
<b>Experiment III</b>	
Control	35±14
10 nM TPA	2,250±165
10 nM TPA + 10 $\mu\text{M}$ H-7	1,860±167
10 nM TPA + 50 $\mu\text{M}$ H-7	1,580±208
10 nM TPA + 100 $\mu\text{M}$ H-7	894±172

Cells were cultured and TPA added as described in Fig. 1. 60 min before addition of TPA, TFP, ALP, CP, or H-7 at the indicated concentrations dissolved in 50 or 30  $\mu\text{l}$  PBS were added to parallel cultures. 11 h later the cultures were incubated with 10  $\mu\text{M}$  AA and 60 min later the concentration of TXB<sub>2</sub> was determined as described in Methods. It is noteworthy that TFP at 50  $\mu\text{M}$  almost completely prevented HL-60 cell adhesion, ALP at 1  $\mu\text{M}$  only moderately prevented HL-60 cell adhesion assayed after 24 h whereas 5  $\mu\text{M}$  almost completely prevented HL-60 cell adhesion. CP at 1  $\mu\text{M}$  reduced TPA-induced HL-60 cell adhesion by 50% whereas it completely prevented HL-60 cell adhesion at 5  $\mu\text{M}$ . Likewise, H-7 at 10  $\mu\text{M}$  had a small effect although it strongly inhibited HL-60 cell adhesion at 100  $\mu\text{M}$ . TFP up to 50  $\mu\text{M}$ , ALP and CP up to 5  $\mu\text{M}$ , and H-7 up to 100  $\mu\text{M}$  did not compromise viability as determined by exclusion of trypan blue 24 h after addition of TPA. TFP, ALP, CP, and H-7 did not interfere with radioimmunologic determination of TXB<sub>2</sub>. The numbers represent means of duplicate dishes±standard deviation.

We therefore tested whether ALP and CP were able to inhibit TPA-induced TXB<sub>2</sub> synthesis as well as macrophage differentiation. Both compounds largely prevented TPA-induced macrophage differentiation and TXB<sub>2</sub> synthesis in a concentration dependent manner (Table II). The isoquinolinesulfonamide, H-7, has been shown to reversibly inhibit both cyclic AMP-dependent protein kinase and PKC by competing with substrate ATP (36). H-7 also inhibited both TPA-induced macrophage differentiation and TXB<sub>2</sub> synthesis (Table II).

The stimulation of TXB<sub>2</sub> synthesis by TPA and diC8 is associated with induction of PGH synthase. The synthesis of TXB<sub>2</sub> that occurred early during macrophage differentiation of HL-60 cells required translational activity because 10  $\mu\text{g}/\text{ml}$  cycloheximide completely prevented the diC8- and TPA-dependent synthesis of TXB<sub>2</sub> (Table III). This same dose of the inhibitor blocked by > 95% the incorporation of labeled leucine into cell proteins and also prevented diC8-dependent cell adhesion. To determine whether TPA or diC8 could have specifically induced the formation of PGH synthase, we first determined whether the rate of enzyme formation in TPA-treated cells was sufficient to account for the rapid increase in TXB<sub>2</sub> synthesis observed in the time course experiment shown in Fig. 3. Evidence for this was provided by experiments with HL-60 cells that had been pretreated with aspirin to block irreversibly the activity of the PGH synthase (37). When we subsequently removed the aspirin, then treated the cells with TPA, we found that a significant stimulation of TXB<sub>2</sub> synthesis occurred within 1 h and that a stimulation equivalent to that observed in TPA-treated control cells occurred within 4–6 h (Fig. 7).

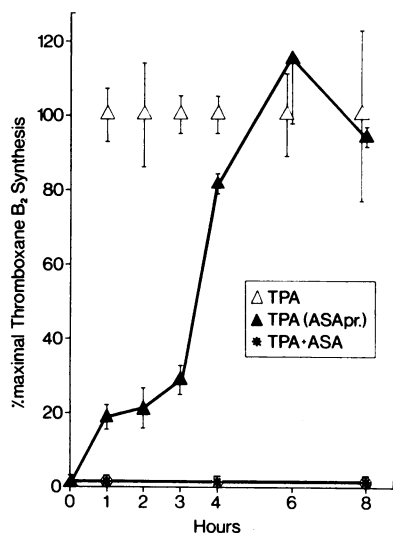
To obtain more direct evidence for a stimulatory effect of TPA and diC8 on the PGH synthase, we both measured the activity of the enzyme and estimated the mass of the enzyme in cell-free preparations. When we measured the kinetics of synthesis of TXB<sub>2</sub>, PGE<sub>2</sub>, and 6-keto-PGF<sub>1 $\alpha$</sub>  from exogenous AA in microsomes prepared from undifferentiated and cells that had been induced to differentiate into macrophages either by TPA or diC8, we observed large increases in the  $V_{\text{max}}$  values in differentiating cells as compared with control cells, but no changes in  $K_m$  values (Fig. 8, Table IV, results not shown).

When we estimated the mass of the enzyme by using two

Table III. TPA- and diC8-dependent TXB<sub>2</sub> Synthesis from Exogenous AA Requires Translational Activity

Additions	TXB <sub>2</sub> pg/10 <sup>6</sup> cells
Control	105±25
10 nM TPA	1,990±160
10 nM TPA + 10 $\mu\text{g}/\text{ml}$ cyclo	188±95
0.1 nM TPA	195±72
30 $\mu\text{M}$ diC8	390±60
30 $\mu\text{M}$ diC8 + 0.1 nM TPA	2,480±190
30 $\mu\text{M}$ diC8 + 0.1 nM TPA + 10 $\mu\text{g}/\text{ml}$ cyclo	240±125

Cells were cultured as described in Fig. 1. 10  $\mu\text{g}/\text{ml}$  cycloheximide was added in 50  $\mu\text{l}$  of Dulbecco's PBS 30 min before addition of diC8 and TPA. At 5 h parallel cultures were incubated with 10  $\mu\text{M}$  AA for 60 min and the concentration of TXB<sub>2</sub> was determined as described in Methods. Inclusion of cycloheximide (cyclo) in the culture medium did not compromise viability of the cells as judged by exclusion of trypan blue or release of LDH. Numbers represent means of duplicate dishes±standard deviation.



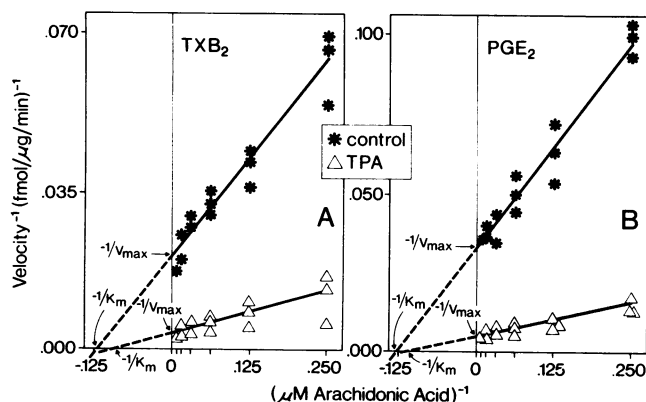
**Figure 7.** Recovery of TXB<sub>2</sub> synthesis from exogenous AA after inhibition of PGH synthase by aspirin. Cells were cultured as described in Fig. 1. Before addition of TPA, cells were incubated with 100 μM aspirin (ASA) dissolved in a concentrated solution of 50 μl PBS for 60 minutes. Thereafter aspirin-treated and control cells were washed twice using 5 ml PBS, and incubated with TPA as described in Fig. 1. 60 min before the time points indicated in the figure,

the cells were incubated with 10 μM AA and the concentration of TXB<sub>2</sub> was determined as described in Methods. The data points represent means of triplicate determinations ± standard deviation.

monoclonal antibodies directed against different antigenic sites on sheep seminal vesicle PGH synthase, we were unable to detect measurable amounts in undifferentiated cells. In contrast, the monoclonal antibodies detected significant amounts of PGH synthase in HL-60 cells that had been induced to differentiate by TPA (Table V).

## Discussion

Many studies have shown that both adherent, blood-derived monocytes and tissue macrophages can respond to immune complexes and other stimuli by releasing unesterified AA from endogenous phospholipids and then converting the AA into icosanoids (38–42). Such cells seem to form preferentially TXB<sub>2</sub>, PGE<sub>2</sub>, and leukotriene B<sub>4</sub>. There is evidence also that the release of AA from monocytes/macrophages may be rate limiting for icosanoid biosynthesis. For example, in response to opsonized



**Figure 8.** Kinetic parameters of microsomal TXB<sub>2</sub> and PGE<sub>2</sub> synthesis in control and TPA-treated HL-60 cells. Cells were cultured and TPA added as described in Fig. 1. After 12 h microsomes from control and TPA-treated cells were prepared and microsomal (A) TXB<sub>2</sub> synthesis and (B) PGE<sub>2</sub> synthesis were determined as described in Methods. The results were subjected to linear regression analyses. The data points indicated in the figure represent means of duplicate determinations in each of three independent experiments.

**Table IV.** Comparison between the Kinetic Parameters of TXB<sub>2</sub> Synthesis in Microsomes Prepared from Control HL-60 Cells and Cells Treated with diC8 and/or TPA

Additions	TXB <sub>2</sub>	
	V <sub>max</sub> fmol/μg protein per min	K <sub>m</sub> μM AA
Control	47.5±5.9	8.0±2.6
10 nM TPA	253.3±57.4	7.4±3.3
0.1 nM TPA	57.9±10.6	10.8±1.7
0.1 nM TPA + 30 μM diC8		
single dose	295.4±33.3	8.4±1.5
70 μM diC8 single dose	90.0±15.0	8.6±3.2
30 μM diC8 loading dose + 4 × 10 μM diC8		
maintenance doses hourly	376.0±22.1	9.2±2.7

Cells were cultured and TPA was added as described in Fig. 1. diC8 was added as a single dose or repeatedly (four times at hourly intervals) as described in Fig. 6. 12 h after the first addition of diC8 or TPA, microsomes were prepared and assayed for PGH synthase activity as described in Methods and Fig. 8. The data represent means of three experiments ± standard deviation.

zymosan, mouse peritoneal macrophages release unesterified AA and synthesize PGs after a lag time of ~ 15 min (40). In contrast, similar cells, incubated in the absence of a particulate stimulus, but in the presence of added unesterified AA or Ca<sup>2+</sup> ionophore, apparently produce icosanoids without delay (43). These and other results have led to the concept that the induction of icosanoid synthesis in macrophages requires the activation of phospholipases, but may not require the activation or induced synthesis of enzymes that convert unesterified AA into oxidized products (44).

The important possibility remains, however, that the activity of enzymes, such as PGH synthase, that directly catalyze the formation of icosanoids, may be tightly regulated during one or

**Table V.** Estimation of HL-60 PGH Synthase Mass by Use of Cross-reacting Monoclonal Antibodies Directed against Sheep Seminal Vesicle PGH Synthase

Additions	Synthase mass
	pg/μg HL-60 microsomal protein
Control	Not detectable
10 nM TPA	1,190±350

Cells were cultured and TPA added as described in Fig. 1. 12 h after addition of TPA, microsomes of control and TPA-stimulated cells were prepared as described in Methods and assayed for the presence of PGH synthase using two monoclonal antibodies (cyo-1, cyo-5) directed against two antigenic sites on sheep seminal vesicle PGH synthase that cross-react with the human enzyme (22, 23) as described in Methods. To generate the standard curve, partially purified sheep seminal vesicle PGH synthase was used. The results are reported as picograms of standard PGH synthase per microgram of HL-60 microsomal protein. The data represent means of two assay samples ± standard deviation. Similar results were obtained in a second experiment.

more stages of macrophage "activation" or development. Regulation of these enzymes might explain the observation (45) that immunological "activation" of macrophages is associated with preferential synthesis of TXB<sub>2</sub> at the expense of PGE<sub>2</sub>. In addition, regulation of enzymes of icosanoid biosynthesis might be presumed to occur at some stage during macrophage development because the icosanoid patterns of monocytes/macrophages differ considerably from those of other terminally differentiated white blood cells that are derived from the same pluripotent stem cells in the bone marrow (5, 39, 41, 42, 44, 46, 47).

The present study was performed to explore the possibility that enzymes that directly catalyze the synthesis of icosanoids may be upregulated during the differentiation of promyelocytic cells into macrophages. In initial experiments we treated the HL-60 cells with TPA or diC8 to initiate their differentiation into macrophages, then measured the synthesis of TXB<sub>2</sub> in the presence of added unesterified AA. The rationale for adding exogenous unesterified AA to the cells was to allow icosanoid synthesis to proceed independently of the release of endogenous AA from cellular phospholipid stores. The results of these experiments support several main conclusions. First, unlike macrophages, undifferentiated HL-60 cells form very little TXB<sub>2</sub> in response to either added unesterified AA or added Ca<sup>2+</sup> ionophore (Figs. 1 and 2, Table I). These results are consistent with previous data obtained in HL-60 cells induced to differentiate into macrophages by (OH)<sub>2</sub>vitamin D<sub>3</sub> (48). Secondly, TPA-stimulated cells show an increased capacity to form TXB<sub>2</sub> and two other products of the PGH synthase pathway, PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub> , early during the course of their differentiation into macrophages (Table I). Thirdly, this response probably depends on the activation of PKC because the only phorbol diesters that are effective are those that stimulate this enzyme (not shown), because diC8 is effective (Figs. 4–6), and because several different inhibitors of PKC prevent both the differentiation of HL-60 cells into macrophages and the increased ability of the cells to synthesize TXB<sub>2</sub> (Table II). These results are consistent with the recent report of Merrill et al. (57) indicating that phorbol diester- and diC8-dependent HL-60 macrophage differentiation is inhibited by the novel PKC inhibitor sphinganine, which may function physiologically as a negative effector of this enzyme. In subsequent experiments, we provided strong evidence that the increased ability of the HL-60 cells to synthesize TXB<sub>2</sub>, PGE<sub>2</sub>, and 6-keto-PGF<sub>1 $\alpha$</sub>  depends on an induced biosynthesis of PGH synthase. This evidence was based on the following observations: (a) both added unesterified AA and added Ca<sup>2+</sup> ionophore greatly promoted the synthesis of TXB<sub>2</sub> in differentiating cells but not in undifferentiated cells (Figs. 1–3); (b) the effects of TPA or diC8 on TXB<sub>2</sub> synthesis became evident after a lag period that was comparable with that shown by cells that were recovering from the effects of pretreatment with aspirin (Figs. 3 and 7); (c) the effects of TPA or diC8 on TXB<sub>2</sub> synthesis were prevented by cycloheximide (Table III); (d) microsomes from TPA- or diC8-treated cells showed increased activity of PGH synthase (Fig. 8, Table IV); and, most importantly, (e) immunoassays of PGH synthase in TPA-treated cells demonstrated a marked increase in enzyme protein (Table V). We conclude, therefore, that PGH synthase is upregulated during the differentiation of HL-60 cells into macrophages.

Four questions remain, however: (a) What is the mechanism of the effect of PKC on the induction of PGH synthase? (b) Is the PGH synthase response part of a coordinated response in-

volving several enzymes of icosanoid biosynthesis? (c) How does the enzyme response in differentiating macrophages differ from responses that may occur in other differentiating white blood cells? (d) What is the functional significance of the response? Only partial answers to these questions can be given at present.

It is unclear whether PKC directly affects a specific step in the biosynthesis of PGH synthase or whether its effects on PGH synthase are only indirect. What does seem clear is that the effects of PKC on HL-60 cell differentiation and icosanoid synthesis are closely linked. Moreover, it appears that prolonged activation of the kinase may be required for both types of effect. This is suggested by the fact that a single dose of diC8, even at a high concentration, failed to induce maximal production of TXB<sub>2</sub> or maximal adherence of the cells to the culture dish. In contrast, both effects occurred when a loading dose of diC8 was followed at hourly intervals by four successive maintenance doses of diC8 (Fig. 6). This is consistent with the work of Ebeling et al. (13) who showed that repeated addition of diC8 to HL-60 cells is required to maintain occupancy of the phorbol diester binding sites and induce macrophage differentiation. Interestingly, we found that a single dose of diC8 was nevertheless effective when added in conjunction with a subthreshold dose of TPA (Figs. 4 and 5). The basis for this synergism remains to be clarified. It is probable that a single dose of diC8 acts only transiently because it is rapidly metabolized. It is known, for example, that HL-60 cells can rapidly convert diC8 into phosphatidic acid (18), and that TPA competitively inhibits diacylglycerol lipase activity (49). One possibility, therefore, is that these reactions or others that metabolize diC8 might be affected by TPA. Alternatively, TPA might promote the accumulation of endogenous diacylglycerol or potentiate the effects of diC8 by binding to cellular sites other than PKC.

As for the possibility that macrophage differentiation might be accompanied by the coordinately controlled upregulation of several enzymes in the icosanoid biosynthesis pathway, and the possibility that different patterns of coordinate control might operate during the differentiation of different types of white blood cells, little can be said at present except for the following: First, our observation that the synthesis of three different icosanoids increased during the differentiation of HL-60 cells clearly raises the possibility that several different enzymes were upregulated in concert. Indeed, we observed greatly increased synthesis of TXB<sub>2</sub> from PGH<sub>2</sub> in differentiating, but not in undifferentiated cells (manuscript in preparation). Secondly, preliminary experiments with HL-60 cells that were induced to differentiate into granulocytes have provided evidence that the granulocyte- and macrophage-differentiation pathways are associated with different TXB<sub>2</sub> responses. When we induced differentiation of HL-60 cells into granulocytes by treating the cells with dimethyl sulfoxide (DMSO) (50) for 5 d, we found by morphologic examination that > 95% of the cell population had differentiated along the myeloid pathway: promyelocytes, 3% (control 95%); myelocytes, 36.5%; metamyelocytes, 36.5%; stab cells, 13.5%; polymorphonuclear leukocytes, 10.5%. Furthermore, the cells showed an increased synthesis of TXB<sub>2</sub> in the presence of added unesterified AA, as has been reported by others (51). However the effect of DMSO on TXB<sub>2</sub> synthesis was relatively small, reaching only 25% of that of TPA. In addition, the kinetics of TXB<sub>2</sub> synthesis were different: whereas treatment with TPA resulted in maximal synthesis of TXB<sub>2</sub> within 3–6 h, treatment with DMSO induced maximal synthesis rates within 24 h. Retinoic acid, another inducer of granulocyte differentiation (52),



was even less active than DMSO in stimulating TXB<sub>2</sub> synthesis within the same period of time. Bonser et al. (53) and Anthes et al. (54) have previously shown that myeloid differentiation of HL-60 cells is associated with induction of the 5-lipoxygenase pathway and Lundberg et al. (55) demonstrated the appearance of the 15-lipoxygenase pathway in the same system. Experiments performed in our laboratory (unpublished observations) confirmed these previous results and extended them by demonstrating the appearance of the 5-lipoxygenase pathway in both the myeloid and the macrophage differentiation pathways. Further work will clearly be required to characterize the precise patterns of icosanoid biosynthesis in the granulocyte- and macrophage-differentiation pathways and to show whether additional differences between the two types of responses exist.

Further work will also be required to establish the functional significance of icosanoid formation during macrophage differentiation. HL-60 cells are transformed cells and thus only models of normal promyelocytes. Furthermore, we used two unphysiological substances, TPA and diC8 to induce their differentiation. It will be important, therefore, to determine whether normal bone marrow promyelocytes respond to physiological differentiation factors by upregulating enzymes of icosanoid biosynthesis. A physiologic response system of this type might be particularly useful in the investigation of potential autocrine and paracrine effects of icosanoids. Indeed, Kurland and his colleagues (56) have already provided evidence that PGs formed by adherent monocytes can inhibit colony formation by granulocyte/macrophage stem cells derived from normal human bone marrow.

In summary, our results demonstrate that the differentiation of HL-60 cells into macrophages is associated with an early upregulation of PGH synthase. They show that this upregulation depends on induction of enzyme synthesis, and support the conclusion that formation of PGH synthase contributes critically to the regulation of the synthesis of TXB<sub>2</sub>, PGE<sub>2</sub>, and 6-keto-PGF<sub>1α</sub>. They suggest that activation of PKC for ~ 5 h is required for both the maximal induction of macrophage differentiation and the maximal induction of icosanoid biosynthesis. More work is needed now to elucidate the mechanism and functional significance of these effects.

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