Increased synthesis of insulin-like growth factor-1 is induced in murine macrophages by prostaglandin E2 (PGE2) and tumor necrosis factor-α (TNFα). Accordingly, we have investigated mechanisms regulating synthesis of PGE2 that might contribute to autocrine/paracrine effects on insulin-like growth factor-1 production. In response to zymosan, TNFα specifically induced a 5-fold increase in PGE2 synthesis, at the same time decreasing PGD2 production in a reciprocal fashion. Activators of cyclic AMP-dependent protein kinase (PKA), such as PGE2 itself or dibutyryl cyclic AMP, did not modify PGE2 production by themselves but potentiated the TNFα-induced increase in PGE2 this effect required both RNA and protein synthesis. No significant change in arachidonate release or production of other eicosanoids was observed. The inducible form of cyclooxygenase-2 (COX2) but not of the constitutive form COX1 was implicated in the generation of both PGE2 and PGD2 in these cells by use of specific inhibitors and effects of dexamethasone. Neither COX1 nor COX2 protein levels were affected by TNFα or PKA activators used alone, whereas in association, marked up-regulation of COX2 mRNA and protein was observed. Incubations of cells carried out with PGH2 demonstrated that PGE2 synthase activity was increased after a TNFα pretreatment. Taken together, our results suggest that TNFα induced a switch from the PGD2 to PGE2 synthesis pathway by regulating PGE2 synthase expression and/or activity and that activators of PKA markedly potentiated the TNFα-induced increase in PGE2 through up-regulation of COX2 gene expression.

Macrophages are known to generate prostaglandins (PGs)1 in response to various stimuli such as endotoxin (1, 2), phorbol myristate acetate (3), or phagocytic particles (4). In addition to playing important roles in such biologic processes as cell proliferation (5), inflammatory and immune responses (6–8), and the production of extracellular matrix proteins (9), prostaglandins may also act in an autocrine/paracrine fashion to modulate the responses of the macrophages themselves (10–12). We have previously shown that in murine macrophages, prostaglandin E2 (PGE2) increased the synthesis of insulin-like growth factor-1 (IGF-1), a growth factor for fibroblasts, by a TNFα-induced process (13). Since macrophages are themselves a potent source of PGE2, autocrine up-regulation of IGF-1 was a distinct possibility. Furthermore, TNFα might itself alter the IGF-1 response by, in part, enhancing production of PGE2. Accordingly, in this study, we addressed the effects of TNFα and PGE2 on PGE2 production in murine bone marrow-derived macrophages. Since PGE2 acts by receptor-mediated generation of cyclic AMP and activation of protein kinase A (PKA), the effect of direct addition of dibutyryl cyclic AMP (dbcAMP) was also examined.

The first enzymatic step in eicosanoid synthesis is the release of free arachidonic acid (AA) from membrane phospholipid. Depending on the cell type and stimulus, different forms of phospholipase A2 (PLA2), including an 85-kDa cytosolic PLA2 (cPLA2) (14, 15), 14-kDa secreted group II PLA2 (sPLA2) (16–18), or a calcium-independent cytosolic PLA2 (19, 20), have each been described to play a role in mediating AA release and subsequent prostaglandin synthesis. Agents that stimulate AA release in macrophages, including zymosan, phorbol 12-myristate 13-acetate, calcium ionophore A23187, and okadaic acid, can activate cPLA2 by enhancing serine phosphorylation of the enzyme (21). More recently, a role for mitogen-activated protein kinase has been described in mediation of agonist-induced activation of cPLA2 (22, 23). Cytokine-induced changes in gene expression of sPLA2 have been reported in rat mesangial cells (24) and human synovial cells (25), but the activity of these enzymes is presumably also regulated by the extent and location of their secretion.

Prostaglandin endoperoxide-synthase or cyclooxygenase (COX) catalyzes the conversion of AA to PGH2, which is then metabolized by one or more terminal synthases to a variety of biologically active prostanooids (26). Cyclooxygenase is a key enzyme in prostanoid synthesis and possesses both fatty acid cyclooxygenase activity (producing PGG2 from AA) and PG hydroyperoxidase activity (converting PGG2 to PGH2). COX2 is a recently described form of cyclooxygenase that is induced in a number of cell types by proinflammatory stimuli, which contrasts to the lack of induction seen with the previously characterized constitutive enzyme, COX1 (27). Thus, COX2 is thought to contribute to the generation of prostanooids at sites of inflammation (28), (see Ref. 29 for review).
In the current study, the ability of murine macrophages to synthesize PGE₂ in response to a phagocytic particle (zymosan) was selectively induced by TNFα. In contrast, a concomitant decrease in PGD₂ production was observed after TNFα priming, suggesting a striking switch from production of PGD₂ to PGE₂ and regulation of PG synthase activity. In addition, we show herein that activators of PKA markedly potentiated the TNFα-induced increase in PGE₂ through up-regulation of COXI gene expression. Our results suggest that (i) PGE₂ might act as an autocrine mediator to stimulate and to maintain the differentiation of uncommitted macrophages into IGF-1 producing cells and (ii) this differentiated cell type may participate in an excocrine fashion to regulate the inflammatory response by its ability to synthesize and release PGE₂.

EXPERIMENTAL PROCEDURES

Animals—C57/Hoe mice were bred in the Biological Resource Center at the National Jewish Center. The C3H/Hoe strain is lipopolysaccharide hyporesponsive and was chosen to minimize the effects of trace lipopolysaccharide contamination (30). Approximately 8-week-old mice were fed CO2 narcosis.

Materials—Dubeclo’s modified Eagle’s medium was obtained from Bio-Whittaker (Walkersville, MD). Fetal bovine serum (Hybri-Max) was prepared as the indicated period of time with various stimuli, the cell monolayers were scraped in 500 μl of 0.5 N sodium hydroxide, and total protein was measured using a BCA protein assay. 

ULTIMATE Bone Marrow-Derived Macrophages—Murine bone marrow-derived macrophages were obtained using a technique previously described in detail (31). Dubeclo’s modified Eagle’s medium was prepared by incubating 12.4 mM glutamine, 0.56% (w/v) NaHCO₃, 10% (v/v) heat-inactivated fetal bovine serum, and 1% (v/v) L929 cell-conditioned medium overnight. The bone marrow cells were flushed aseptically from the dissected pelvis, femurs, and tibias of C57/Hoe mice with a jet of complete medium directed through a 25-gauge needle to form a single cell suspension.

The bone marrow cells were centrifuged at 200 g for 1 min, and reduced glutathione (6 μM in 0.1% (v/v) methanol) was added for 3 min. The reaction was terminated by adding 10 μl of a 25 mM FeCl₃ solution. Spontaneous conversion was evaluated by incubating PGH₂ in buffer alone with no cell. The concentrations of PGE₂ and PGD₂ at 0 and 1 min were determined by enzyme immunoassay.

Quantification of mRNA Transcripts—The expression of COX1, COX2, and GAPDH mRNA transcripts was determined by Northern analysis. Total cellular RNA was extracted from macrophage monolayers with 1 ml of RNAzol B for 1.8 h (30). 10 μg of total RNA was electrophoresed under denaturing conditions through a 1.2% agaroseformaldehyde gel and then transferred to NYTRAN hybridization filters as described (39). Total RNA was covalently linked to the membrane by UV cross-linking (10 joules) using a Strataphen 1800 (Stratagene, La Jolla, CA). The mRNA was hybridized with 10^6 dpm/ml of 32P-labeled cDNA probes for 18 h and washed to a final stringency of 0.2 × SSC at 42 °C, and autoradiograms were prepared by exposure to Kodak X-OMAT AR film at −70 °C. To ensure that the differences in transcript expression were specific to COX1 and COX2 mRNA and not due to differential loading of RNA, the blots were stripped with 2% (w/v) SDS and reprobed with GAPDH. The density of the autoradiograms and the signals was quantitated by scanning densitometry and normalized with integration using the Image 1.35 software run on a Macintosh microcomputer. The results were expressed as a ratio of COX to GAPDH expression as indicated.

SDS-Polyacrylamide Gel Electrophoresis/Immunoblot Analysis—Macrophage monolayers were scraped into ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 1% Nonidet P-40, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, and 1 mM aprotinin. 50 μg of total protein determined using a BCA protein assay was separated in SDS/10% polyacrylamide gels and transferred onto nitrocellulose membranes. The blots were washed in Tris-buffered saline (20 mM Tris, pH 7.6, 137 mM NaCl) with 0.05% (v/v) Tween 20 (TBST), blocked overnight with 5% (w/v) fat-free dry milk in TBST. The same blot was probed with a monoclonal antibody for COX1, stripped, and then reprobed with a monoclonal antibody for COX2.

Data Analysis—Results are presented as the mean ± S.E. for at least three separate experiments. Comparisons between groups were made using the student’s paired t test.

RESULTS

Effect of TNFα, PGE₂, and dbcAMP on PGE₂ Synthesis and Release—Murine bone marrow-derived macrophages were cultured for 5 days in the presence of colony-stimulating factor-1, followed by priming with TNFα, PGE₂, or dbcAMP alone or in combination for a period of 8 h. After the culture medium was removed, macrophages were washed with PBS, incubated in 100 μl of media at 37 °C for 1 h with 50 particles per cell of opsonized zymosan in fresh culture medium to promote production and release of arachidonic acid metabolites. Previous analysis had shown this amount and time of exposure to zymosan to be optimal (data not shown). As shown in Fig. 1, 10 ng/ml TNFα led to a 5-fold increase in PGE₂ production, whereas no change was observed.
in response to either 0.1 mM dbcAMP or 1 μM PGE₂ treatment alone. However, when cells were incubated with the combination TNFα+dbcAMP or TNFα+PGE₂, synergistic responses were observed corresponding to a 4-fold increase and a 2-fold increase compared with TNFα alone for dbcAMP and PGE₂, respectively.

**Release of Free Arachidonic Acid**—To determine the point at which the TNFα and PGE₂ were exerting their effect, the release of free AA was analyzed to explore the possibility that these effectors may affect the balance of deacylation and recacylation. To block metabolism of free AA released by COX and 5-lipoxygenase (5-LO), cells were incubated in the presence of 5 μM indomethacin to block the COX activity (40) and 5 μM zileuton to inhibit 5-lipoxygenase (41) for the 1-h stimulation period with zymosan. In Fig. 2, intracellular plus extracellular AA content is shown, as determined by mass spectrometry and expressed as nanogram/microgram of protein; values represent the mean ± S.E. of three to six independent experiments. *, p < 0.05.

**Effect of TNFα, PGE₂, and dbcAMP on Synthesis and Release of Other Eicosanoids**—To determine whether the observed induced increase in PGE₂ production was exclusive to PGE₂, the effect on other AA metabolites known to be produced by macrophages was examined. The production of two cyclooxygenase metabolites, TXB₂ and 6-keto-PGF₁α (the stable PGI₂ metabolite), as well as two 5-lipoxygenase products (LTC₄ and LTB₄), were quantified in the supernatant of cells primed for 18 h with zymosan. In Fig. 2, intracellular plus extracellular AA content is shown, as determined by mass spectrometry and expressed as nanogram/microgram of protein; values represent the mean ± S.E. of three to six independent experiments. *, p < 0.05.

**Requirement of Protein and RNA Synthesis in PGE₂ and PGD₂ Production**—To further investigate the molecular basis involved in the regulation of PGE₂ and PGD₂ synthesis in murine macrophages, cells were treated with 10 ng/ml TNFα alone or in combination with 0.1 mM dbcAMP in the presence or absence of 5 μg/ml cycloheximide or 0.5 μg/ml actinomycin D. As illustrated in Fig. 4, the observed increase in PGE₂ production after stimulation of cells with TNFα alone or in combination with dbcAMP was completely abolished by either cycloheximide or actinomycin D, suggesting an absolute requirement for both RNA and protein synthesis. In contrast, neither cycloheximide nor actinomycin D inhibited basal PGD₂ levels. These results suggest that, unlike the TNFα+dbcAMP-mediated induction of
PGE2 synthesis, basal production of PGD2 does not require new RNA synthesis. The observed decrease in PGD2 synthesis following cycloheximide treatment might reflect the turnover of constitutive enzyme(s) involved in the basal production of this prostaglandin.

**TNFα and dbcAMP Synergistically Up-regulate COX2 Gene Expression and Protein Synthesis**—Since protein and RNA synthesis were involved in the enhanced production of PGE2 by the combination of TNFα + dbcAMP and because basal production of PGD2 was dependent of protein synthesis, we next examined steady state mRNA levels of the constitutive (COX1) and the inducible form (COX2) of cyclooxygenase. Messenger RNA levels were analyzed by Northern blot after a 12-h stimulation period, and hybridization with a GAPDH cDNA probe was performed to ensure for equal loading of total RNA. As shown in Fig. 5, COX1 mRNA was constitutively expressed in control macrophages while COX2 mRNA level remained undetectable. Neither 1 μM PGE2 nor 0.1 mM dbcAMP affected the expression of either COX1 or COX2, whereas 10 ng/ml TNFα inhibited the basal expression of COX1 mRNA and seemed to slightly increase COX2 mRNA levels. When cells were incubated with the combination TNFα + PGE2 or dbcAMP, elevation in COX2 mRNA levels was observed. The TNFα-induced inhibition of COX1 mRNA expression was not significantly altered by the presence of PGE2 or dbcAMP. As seen in Fig. 6, Western blot analysis of macrophage whole cell lysates

**Table I**

|          | TXB2 | 6-keto-PGF1α | LTC4 | LTB4 |
|----------|------|--------------|------|------|
| Untreated| 12.95 ± 1.86 | 0.46 ± 0.04 | 1.29 ± 0.54 | 0.33 ± 0.03 |
| 10 ng/ml TNFα | 7.91 ± 1.20 | 0.88 ± 0.51 | 0.717 ± 0.39 | 0.29 ± 0.11 |
| 0.1 mM dbcAMP | 16.93 ± 3.09* | 1.10 ± 0.80 | 1.74 ± 0.98 | 0.56 ± 0.05* |
| TNFα + dbcAMP | 10.57 ± 0.83 | 1.95 ± 1.24 | 0.78 ± 0.46 | 0.71 ± 0.25 |

* *p < 0.05 compared with values of untreated.

**Fig. 3. Regulation of PGE2 and PGD2 production by TNFα.** A, cells were incubated with increasing concentrations of TNFα for 16 h and stimulated for 1 h with zymosan. Results of one representative experiment (out of three) are expressed in picograms of prostaglandin per microgram of protein. B, macrophages were treated with 10 ng/ml TNFα for 16 h and stimulated for 1 h with 10 μM exogenous AA. Results are expressed as pg/μg and represent the mean ± S.E. of three (PGD2) or four (PGE2) experiments. *, p < 0.05.

**Fig. 4. Effect of inhibiting RNA and protein synthesis on PGE2 and PGD2 production.** Macrophages were treated with 10 ng of TNFα alone or in combination with 0.1 mM dbcAMP in the presence or absence of 5 μg/ml CHX or 0.5 μg/ml AD. At 16 h, cells were stimulated for 1 h with zymosan. Results are expressed as a percentage of the value in cells incubated without any inhibitors and represent the mean ± S.E. of three separate cultures. Student’s paired t test was used to compare TNFα or TNFα + dbcAMP-treated versus untreated (plain bars) and CHX or AD-treated versus control. *, p < 0.05.
using specific monoclonal antibodies for COX1 and COX2 showed that the COX2 protein levels paralleled those of the transcript. The low content of COX2 protein in untreated cells was not affected by any of the stimuli used alone but was enhanced when cells were incubated with the combination of TNFα1dbcAMP. In contrast, the basal expression of COX1 protein remained unchanged in response to any of the treatments. Although the TNFα-induced down-regulation of COX1 mRNA levels was observed at 12 h, no significant change in the corresponding protein was seen after 18 h. This latter observation suggests that the constitutive form of COX may be relatively stable so that protein levels were not yet affected at 18 h despite the absence of transcript at 12 h.

Involvement of COX2 Activity in Both PGE2 and PGD2 Productions—To further characterize which COX isozyme was involved in the up-regulated synthesis of PGE2, inhibitors of COX1 and/or COX2 activities were used. As shown in Fig. 7A, the COX inhibitor indomethacin totally prevented the TNFα+dbcAMP-induced increase in PGE2 synthesis. The COX2 inhibitor NS-398 (40) duplicated the indomethacin results. However, a concentration 10 times higher (10 μM versus 1 μM) was required. In contrast, neither sulindac sulfide (42) nor valeryl salicylic acid (43), which are 10 times more potent inhibitors of COX1 activity, decreased PGE2 levels even when used at 10 μM. Similar patterns of inhibition were obtained when cells were primed with TNFα alone (data not shown). To determine whether the basal levels of PGD2 were produced as a consequence of COX1 or COX2 activity, PGD2 release was analyzed in untreated macrophages incubated with increasing concentration of the inhibitors used above. Results shown in Fig. 7B suggest that COX2 and not COX1 is involved in PGD2 synthesis despite the low basal amounts of both COX2 mRNA and protein.

Glucocorticoids have been reported to prevent the induction of COX2 (44, 45). To further examine the possibility that only COX2 was involved in production of PGD2 (before priming) as well as the induced synthesis of PGE2, the effect of steroids on COX1 and COX2 mRNA levels as well as PGE2 and PGD2 production was examined. At 12 h, total RNA was extracted, and the same membrane was consecutively hybridized with COX2, COX1, and GAPDH cDNA probes (Fig. 8). After scanning of the autoradiographs, COX1 and COX2 values were
normalized to the density of the GAPDH band. Dexamethasone prevented the TNFα+dbcAMP-induced increase in COX2/GAPDH mRNA level by 70%, whereas slight increase in steady state levels of COX1/GAPDH mRNA was observed in control. To analyze the effect of glucocorticoids on PGE$_2$ and PGD$_2$ production, cells were primed with stimuli for 16 h as before, but because glucocorticoids inhibit PLA$_2$ activity (46), cells were incubated in the presence of 10 μM exogenous arachidonic acid rather than zymosan to bypass the requirement for PLA$_2$. As depicted in Fig. 9, the presence of 1 μM dexamethasone inhibited both constitutive production of PGD$_2$ (Fig. 9B) and induced increase in PGE$_2$ synthesis (Fig. 9A), suggesting that expression of COX2 is necessary for both the synthesis and release of these two prostaglandins.

**TNFα Increases PGE$_2$ Synthase Activity**—To determine whether PGE$_2$ and/or PGD$_2$ synthase activities were modified following TNFα treatment, conversion of exogenously supplied PGH$_2$ to both PGE$_2$ and PGD$_2$ was examined. As shown in Fig. 10, a 16-h preincubation period with 10 ng/ml TNFα induces a 5.2-fold increase in PGE$_2$ synthase activity after incubation of cell lysate for 1 min with 150 ng of exogenous PGH$_2$. This increase in PGE$_2$ synthesis is similar to the one observed when TNFα-primed cells were incubated with zymosan (Fig. 1). When PGD$_2$ synthase activity was examined, no difference between unstimulated and TNFα-treated macrophages was observed, suggesting that TNFα increases PGE$_2$ synthase activity without affecting PGD$_2$ synthase activity. We next examined the effect of RNA and protein synthesis inhibitors on the activity of the PGE$_2$ synthase (Fig. 11). TNFα-induced increase in PGE$_2$ synthase activity was abolished using either the protein synthesis inhibitor cycloheximide (CHX) or the RNA synthesis inhibitor actinomycin D (AD) or dichlororibofuranosylbenzimidazole. These results suggest that up-regulation of PGE$_2$ synthase activity by TNFα is dependent of both protein and RNA synthesis.

**DISCUSSION**

As shown herein, stimulation of murine macrophages by TNFα induced a switch in prostaglandin synthesis from PGD$_2$ to the PGE$_2$ without affecting production of other eicosanoids. We also demonstrated that PGE$_2$ itself as well as the membrane-permeable PKA activator dbcAMP markedly and selectively potentiated the TNFα-induced increase in PGE$_2$ synthesis. This enhancement could not be explained by increasing activity of PLA$_2$ because no change in AA release was observed. In contrast, we suggest that the enhancing effect of cAMP (PGE$_2$) is mediated through up-regulation of COX2 gene expression. PGE$_2$ inhibits macrophage cytoidal activity and can act as an autocrine-negative feedback stimulus to limit this function (11, 12). In the other direction, we have previously shown that in murine macrophages, PGE$_2$ increased the synthesis of IGF-1 and thus might contribute an autocrine signal to enhance the role of macrophages in wound healing and fibrosis (13). Herein, we show that (i) TNFα selectively induced the production of PGE$_2$ by increasing PGE$_2$ synthase activity and (ii) PGE$_2$ itself up-regulated its own synthesis by TNFα-primed macrophages. These observations help refine the complex, and mutually exclusive, regulatory pathways for synthesis of IGF-1 and for proteins involved in cytoidal macrophage function, respectively (47, 48).

The bone marrow-derived macrophages generated PGD$_2$ as their predominant prostanoid when stimulated to release arachidonate by uptake of zymosan (or upon addition of free arachidonate). In contrast, when incubated with TNFα, the cells switched their production to PGE$_2$ without any significant changes in the release of other eicosanoids from either the 5-lipoxygenase or the cyclooxygenase pathways. The total level of PGs released (PGE$_2$ plus PGD$_2$) was similar in untreated and in TNFα-treated cells. Our results suggest that TNFα induced a switch in PGD$_2$ and PGE$_2$ synthesis from their common precursor PGH$_2$, i.e. that the cytokine was acting at the level of PGD$_2$ and/or PGD$_2$ synthases rather than at the level of active PLA$_2$ or cyclooxygenase. Such a switch has been reported by others in rat Kupffer cells where in vitro activation of protein kinase C resulted in reduced PGE$_2$ and enhanced PGD$_2$ synthase activity (49).

In our study, TNFα did not change the amounts of arachidonate released by the uptake of zymosan. Furthermore, the
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results expressed as ng/ml represent the mean ± S.E. of five different cultures. *, p < 0.05; TNFα versus control at 1 min.

FIG. 10. PGE₂ and PGD₂ synthase activities. Macrophages (2 million) were incubated with or without 10 ng/ml TNFα for 16 h. Exogenous PGH₂ (150 ng) was added to cell lysates, and the tubes were incubated at 37 °C for 1 min in the presence of reduced glutathione. The reaction was stopped with FeCl₃, and PGE₂ and PGD₂ levels were quantified by enzyme immunoassay. Spontaneous conversion of PGH₂ into PGE₂ and PGD₂ obtained in the absence of cells was subtracted from each value. Results are expressed as ng/ml and represent the mean ± S.E. of five different cultures. *, p < 0.05; TNFα versus control at 1 min.

FIG. 11. TNFα-induced increase in PGE₂ synthase activity is dependent on protein and RNA synthesis. Macrophages were incubated with or without 10 ng/ml TNFα for 16 h in the presence or absence of 5 μg/ml CHX, 0.5 μg/ml AD or 50 μg/ml dichlororibofuranosylbenzimidazole (DRB). PGE₂ synthase activity was assayed as described in Fig. 10. Results expressed as ng/ml represent the mean ± variation of two separate experiments.

switch was also evident when arachidonate was added directly without any phagocytic stimulus, i.e. in the absence of obvious PLA₂ activation. Our results also indicate that the TNFα-induced switch to PGE₂ was not mediated through alteration of COX isotype usage. TNFα did not modify either COX1 or COX2 protein levels at 18 h (the time of zymosan addition and prostaglandin measurement) despite a slight increase in COX2 and a decrease in COX1 mRNA steady state levels observed 12 h after TNFα treatment. In addition, use of inhibitors of COX1 and COX2 suggested that COX2 but not COX1 was involved in both the production of PGD₂ before TNFα treatment as well as the PGE₂ formation afterward.

To further investigate the possibility that TNFα might act directly by either increasing PGE₂ synthase activity or by decreasing PGD₂ synthase activity, we incubated cell lysates from control or TNFα-treated cultures with PGH₂ as a substrate before the measurement of prostaglandins. Our results provided evidence that TNFα priming leads to preferential enzymatic conversion of PGH₂ into PGE₂ while PGD₂ generation remains unchanged (Fig. 10). Thus, the observed switch from PGD₂ to PGE₂ does not seem to be mediated by depletion of glutathione following TNFα-induced oxidant stress since both PGE₂ and PGD₂ synthases are glutathione-dependent enzymes (50, 51). We next examined whether the observed TNFα-induced increase in PGE₂ synthase activity was dependent on protein or RNA synthesis. Concomitant incubation of macrophages with TNFα and either cycloheximide or actinomycin D demonstrated that both inhibitors abolished the TNFα-induced effect (Fig. 11). The observation that cycloheximide or actinomycin D prevented the TNFα-induced increase in PGE₂ synthase suggests that TNFα acts through up-regulation of PGE₂ synthase itself or of a regulatory protein (Fig. 4). In addition, TXB₂ levels did not change, suggesting that TNFα acts selectively by inducing PGE₂ activity.

Taken together, our results demonstrate that PGE₂ synthase activity is increased in TNFα-primed macrophages compared with unstimulated cells. The unchanged PGD₂ synthase activity suggests that TNFα does not have any effect at this level. The TNFα-induced switch from PGD₂ to PGE₂ observed in the absence of exogenously added PGH₂ may be the result of preferential use of the endogenous substrate PGH₂ by the PGE₂ synthase pathway.

In previous studies, we showed that in murine macrophages, PKA activators such as PGE₂ and dbcAMP stimulated the synthesis of IGF-1 by a process that was independent of, and additive with, that induced by TNFα (13). As described herein, PKA activators had only a slight enhancing effect by themselves on eicosanoid synthesis in response to zymosan uptake. On the other hand, they did synergistically potentiate the TNFα-induced increase in PGE₂ production. Synergism between cAMP elevating agents and TNFα or interleukin-1β have also been reported in up-regulation of group II PLA₂ mRNA in rat mesangial cells (52–54). Agents that increase cellular levels of cAMP used alone have additionally been shown to increase secretion of PLA₂ from vascular smooth muscle cells (55) and in mesangial cells (54). However, when we examined free AA release by mass spectrometry, no significant change was observed in these macrophages after treatment with PKA activators used alone or in association with TNFα.

However, the combination of TNFα and dbcAMP together did markedly up-regulate COX2 protein and mRNA. Neither stimulus was effective by itself, although, as noted above, TNFα decreased the steady state levels of mRNA for COX1. Incubation of cells with stimulus and cycloheximide or actinomycin D demonstrated that both protein and mRNA synthesis were necessary for the increase in PGE₂ production induced by TNFα plus dbcAMP. Glucocorticoids such as dexamethasone inhibit the induction of COX2 in cultured cells with little effect on COX1 mRNA and protein levels (44, 45). In the current studies, dexamethasone inhibited TNFα plus dbcAMP-induced COX2 mRNA levels as well as PGE₂ generation. The implication that COX2 was required for the PGE₂ synthesis was con-
firmed by use of COX2-selective inhibitors. Importantly, inhibitors of COX2 prevented PGE$_2$ production after TNFα stimulation whether or not the process was further enhanced by dbcAMP. This suggested that COX2 was the critical enzyme involved even without its up-regulation by the combined stimuli. In fact, even the PGE$_2$ produced after zymosan stimulation of macrophages that had not been exposed to either TNFα or PKA activators was prevented by COX2 inhibitors and not by agents more selective toward COX1. All together, our results suggest that TNFα and dbcAMP synergistically up-regulate COX2 gene expression and, as a consequence, provide more substrate (PGH$_2$) for PGE$_2$ production in TNFα-primed macrophages. The synergistic effect of the combination TNFα+dbcAMP on COX2 expression might be explained by the up-regulation of TNFα receptors by agents that increase CAMP levels as it has been described in vitro in human histiocytic lymphoma cell line U-937 (56). Interestingly, we showed herein that TNFα alone leads to a slight increase in COX2 mRNA steady state levels (Fig. 5).

The observed switch from PGD$_2$ to PGE$_2$ synthesis after activation of murine macrophages is biologically relevant because, in these cells, PGD$_2$ did not increase PKA activation or intracellular levels of cAMP (data not shown), although others have reported that it can induce adenylate cyclase activity and PGE$_2$ production in endothelial cells (57). In contrast and as expected, PGE$_2$ was a potent activator of PKA and cAMP generation (13). Thus, PGE$_2$ might be considered to provide a positive feedback stimulus in TNFα-primed macrophages for its own synthesis. Since TNFα by itself induced the switch to PGE$_2$ production by increasing PGE$_2$ synthase activity, the PGE$_2$ so produced would be expected to further enhance its production via up-regulation of COX2 with subsequent positive (e.g. IGF-1 synthesis) or negative (e.g. cytokidal activity) autocrine/paracrine effects on the macrophages.

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