Expression and activity of prostaglandin endoperoxide synthase-2 in agonist-activated human neutrophils

MARC POULIOT,[1] CAROLINE GILBERT, PIERRE BORGEAT,* PATRICE E. POUBELLE,[†] SYLVAIN BOURGOIN,* CHRISTOPHE CRÉMINON,[‡] JACQUES MACLOUF,[§] SHAUN R. McCOLL,[‖] AND PAUL H. NACCACHE[†]

Centre de Recherche en Rhumatologie et Immunologie, Centre de Recherche du CHUQ pavillon CHUL and Laval University, Québec, Canada; Departments of [†]Medicine and *Physiology, Faculty of Medicine, [‡]CEA, SPI/DRM, CE-Saclay 91191 Gif/Yvette CEDEX, France, [§]Inserm U-348, Hôpital Lariboisière, 7547 Paris CEDEX 10, France; and [‖]Department of Microbiology and Immunology, University of Adelaide, Adelaide, South Australia, Australia

ABSTRACT **Proinflammatory agents were assessed for their capacity to stimulate the expression of the inducible cyclooxygenase isoform (COX-2) in human neutrophils. A number of agents, including PMA, opsonized bacteria and zymosan, LPS, GM-CSF, TNF-α, and fMLP, induced COX-2 protein expression through signaling pathways involving transcription and protein synthesis events. Northern blots showed that freshly isolated neutrophils expressed low levels of COX-2 mRNA, which rapidly increased after incubation with inflammatory agents. A characterization of the signal transduction pathways leading to COX-2 protein expression was initiated. In LPS-treated neutrophils, efficient induction of COX-2 required the presence of serum and involved ligand binding to the CD14 surface antigen. The specific inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), SB 203580, had little effect on the induction of COX-2 expression in neutrophils, in contrast to what had been previously observed with other inflammatory cell types. Depending on the agonist present, ethanol differentially blocked the stimulated expression of COX-2, raising the possibility that phospholipase D activation might take part in the process of COX-2 induction. Major COX-2-derived prostanoids synthesized by inflammatory neutrophils were identified by liquid-chromatography and tandem mass-spectrometry as TXA_2 and PGE_2. The agonist-induced synthesis of TXA_2 and PGE_2 was effectively blocked by cycloheximide and by the specific COX-2 inhibitor NS-398. These results show that COX-2 can be induced in an active state by different classes of inflammatory mediators in the neutrophil. They support the concept that, in these cells, the COX-2 isoform is preeminent over COX-1 for the stimulated-production of prostanoids, and also suggest that neutrophil COX-2 displays a distinct profile of expression among circulatory cells.—Pouliot, M., Gilbert, C., Borgeat, P., Poubelle, P. E., Bourgoin, S., Créminon, C., Maclouf, J., McColl, S. R., Naccache, P. H. Expression and activity of prostaglandin endoperoxide synthase-2 in agonist-activated human neutrophils. *FASEB J.* 612, 1109–1123 (1998)**

Key Words: thromboxane · dexamethasone · lipopolysaccharide · cyclooxygenase

NEUTROPHILS ARE the first blood cells that migrate toward inflammatory lesions, where they accumulate in large numbers and perform host defense functions. Well-characterized functions of the neutrophil include the phagocytosis of invading microorganisms and cell debris, the release of proteolytic enzymes, the generation of oxygen-derived reactive agents, as well as the synthesis of cytokines, chemokines (1), and lipid mediators, including leukotriene (LT) B_4, via the lipoxygenase pathway (2). In addition, studies with the hemopoietic factor granulocyte-macrophage colony-stimulating factor (GM-CSF)[2] have illustrated the capacity of neutrophils to up-regulate the expression of specific cellular proteins, an event that may influence the response of these and other immune cells in inflammatory situations (3, 4).

[1] Correspondence: Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesia, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA. E-mail: pouliot@zeus.bwh.harvard.edu

[2] Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; PG, prostaglandins; TX, thromboxane; AA, arachidonic acid; LPS, lipopolysaccharide; COX, cyclooxygenase; PGI_2, prostacyclin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HBSS, Hank's balanced salt solution; RT, room temperature; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC-MS-MS, liquid chromatography and tandem mass spectrometry; fMLP, formyl-methionyl-leucyl-phenylalanine; LBP, LPS binding protein; CX, cycloheximide, AD, actinomycin D, PMA, phorbol 12-myristate 13-acetate; Dex, dexamethasone; COX-2, inducible cyclooxygenase isoform; PAF, platelet-activating factor; TNF-α, tumor necrosis factor α; IL, interleukin; TBS, Tris-buffered saline; EIA, enzyme immunoassay; PLD, phospholipase D; PA, phosphatidic acid; EtOH, ethanol; Indo, indomethacin; MAPK, mitogen-activated protein kinase.

Whereas neutrophils are recognized as a major source of LTB_4, their potential involvement in prostanoid synthesis has been controversial. Though the capacity of human neutrophils to synthesize prostaglandins (PG) and thromboxane (TX) from endogenous arachidonic acid (AA) sources was demonstrated two decades ago (5, 6), few studies since have confirmed these observations (7) and seemingly divergent evidence has accumulated. Incubation of human neutrophils with a number of inflammatory agents, including lipopolysaccharide (LPS), has been reported to lead to the production of PGE_2 in vitro (8); however, it has been suggested that monocytes, but not neutrophils, account for the LPS-triggered cyclooxygenase activity observed in human whole blood ex vivo (9). It remains unclear to what extent neutrophils have the capacity to synthesize cyclooxygenase-derived lipid mediators in response to inflammatory agents.

Prostaglandin endoperoxide synthase, also referred to as cyclooxygenase (COX), catalyzes a cyclooxygenase (*bis*-oxygenase) reaction in which AA is converted to PGG_2 and a peroxidase reaction in which PGG_2 undergoes a two-electron reduction to PGH_2, the common precursor to all prostanoids, including PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and TX (10, 11). Two COX isoforms, encoded by distinct genes (12), have been described in mammalian cells (see ref 13 for a review). The COX-1 isozyme expressed constitutively in most tissues appears to support the levels of prostanoid biosynthesis required for maintaining organ and tissue homeostasis (11, 13). In contrast, the mitogen-inducible cyclooxygenase isoform (COX-2) is the product of an early-response gene that is rapidly induced and tightly regulated (14). COX-2 expression is highly restricted under basal conditions and can be up-regulated during inflammation (15, 16). Although COX-2 is thought to be involved predominantly in the inflammatory response (15, 16), it is also likely to be involved in many essential physiological functions since disruption of the COX-2 gene in mice results in renal dysplasia, cardiac fibrosis, and defects in the ovary (17).

COX-2 expression is restricted to a limited number of cell types (18); it was initially observed in monocytes, vascular smooth muscle cells, synovial cells, and fibroblasts (19). The observation that inhibiting de novo protein synthesis resulted in an inhibition in PGE_2 synthesis in stimulated cells suggested that neutrophils may express an inducible isoform of COX (8). Since then, however, there has been conflicting evidence about the presence of COX-2 in these cells. In separate studies using both in vivo models of inflammation in rat, COX-2 immunoreactivity has either been localized to macrophages, but not to neutrophils (20), or to a fraction of neutrophils and only a small proportion of macrophages (21). Only recently was COX-2 protein expression observed in human neutrophils incubated with LPS (22). The identity of other inflammatory agents that may potentially trigger the expression of this enzyme in human neutrophils remains obscure. Similarly, the mechanisms that regulate the expression of COX-2 in these cells are largely unknown at this time. Furthermore, it remains unclear which, if any, of the two COX isoforms plays a preeminent role in the stimulated synthesis of prostanoids by neutrophils. The aim of this study was to examine the expression of COX-2 in neutrophils and to assess the capacity of classical inflammatory agents to induce this enzyme. We now report that a number of proinflammatory factors can lead to the expression of active COX-2 in human neutrophils.

MATERIALS AND METHODS

Neutrophil preparation

Neutrophil suspensions were prepared as previously described (23), with modifications. Briefly, venous blood collected on citrate dextrose phosphate adenine anticoagulant solution from healthy volunteers was centrifuged (250×*g*, 10 min) and the platelet-rich plasma was discarded. After dextran sedimentation of erythrocytes, polymorphonuclear neutrophils were obtained by centrifugation over a Ficoll-Paque cushion. Contaminating erythrocytes were removed by hypotonic lysis, and purified granulocytes (>95% neutrophils, <5% eosinophils) were resuspended at a concentration of 10×10^6 cells/ml in Hank's balanced salt solution (HBSS) containing 10 mM HEPES pH 7.4, 1.6 mM Ca^{2+}, and no magnesium. Resulting cell suspensions contained fewer than 0.2% monocytes as determined by esterase staining. Viability was greater than 96% as determined by trypan blue dye exclusion. The whole cell isolation procedure was carried out under sterile conditions at room temperature (RT).

Cell incubation and immunoblots

When mentioned, cells were preincubated for 30 min with the appropriate pharmacological agent prepared in either ethanol or dimethyl sulfoxide or with an equal volume of diluent. Organic solvent concentrations never exceeded 0.1% in cell preparations. HBSS was supplemented with 1% fetal calf serum (FCS) for all experiments using LPS. After incubation, cell pellets were resuspended in 150 μl of ice-cold lysis buffer (Hepes-buffered HBSS pH 7.4, 0.5% Triton X-100, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin); 150 μl of 2× sample buffer [1×: 62.5 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 8.5% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.025% bromophenol blue] was added and the mixtures were boiled for 7 min. Samples were then subjected to 9% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon membranes (Millipore Corporation, Bedford, Mass.). Equal protein loading and transfer efficiency was visualized by Ponceau red staining. The membranes were soaked for 30 min at RT in Tris-buffered saline (TBS: 25 mM Tris-HCl pH 7.6, 0.2 M NaCl, 0.15% Tween 20) containing 5% (w/v) dried milk and subsequently exposed to an anti-COX-2 rabbit polyclonal antibody (24). The membranes were washed twice in TBS and incubated with a horseradish peroxidase-linked donkey anti-rabbit antibody (dilution 1:15000). Bound antibodies were revealed with the

enhanced chemoluminescence reagent after the manufacturer's protocol (NEN Life science, Boston, Mass.).

Northern blots

Total RNA was isolated by using Trizol reagent according to the manufacturer's protocol (Gibco-BRL, Grand Island, N.Y.), with modifications. Briefly, 50×10^6 neutrophils were homogenized in 1 ml Trizol and 200 μl of chloroform was added. After a brief mixing by vortex, samples were centrifuged at $12,000 \times g$ for 15 min at 4°C. After centrifugation, 500 μl of the upper aqueous phase was transferred to a tube containing an equal volume of isopropanol. The mixtures were thoroughly vortexed and centrifuged at $12,000 \times g$ for 10 min at 4°C. Supernatants were carefully discarded and the precipitated RNA pellets were washed with 1 ml of 70% ethanol. RNA pellets were centrifuged at $12,000 \times g$ for 5 min at RT. The supernatants were discarded and the pellets were allowed to air-dry for 2–3 min. RNA pellets were resuspended in water and RNA was quantitated by measuring the optical density at 260 nm. The filters were hybridized with a human COX-2 cDNA probe, labeled with [α-^{32}P]dCTP using GIGAprime DNA Labeling Kit (Bresatec, Adelaide, Australia). The COX-2 cDNA probe was synthesized by reverse transcriptase-PCR. The COX-2 primers were 5′-GCT GAC TAT GGC TAC AAA AGC TGG-3′ and 5′-ATG CTC AGG GAC TTG AGG AGG GTA-3′. Integrity of the RNA and equal loading on agarose/formaldehyde gels (10–15 μg/well) were verified by hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The observed COX-2 mRNA band was approximately 4.6 kb, sometimes with two secondary bands of approximately 6 and 3 kb.

Densitometry

Autoradiograms were scanned using a Vista S6E scanner (UMAX Technologies, Inc., Fremont, Calif.) and densitometry was performed using the National Institutes of Health Image program. Densitometry of the doublet corresponding to COX-2 on autoradiograms was evaluated. For each agonist, the value obtained at $t = 4$ h was divided by that obtained at $t = 10$ min in the same experiment in order to account for differences in exposure between experiments.

Analysis of cyclooxygenase products by LC-MS-MS

After appropriate treatment, incubations were terminated by repeated freezing and thawing of the samples, and cell debris was discarded by brief centrifugation ($12,000 \times g$). Samples were acidified to pH 5.0 with acetic acid; 5 ng of PGE_2-D_4 was added as an internal standard. Samples were loaded onto Waters Oasis HLB (60 mg) extraction cartridges (Waters, Milford, Mass.) and the columns were sequentially washed with acidified H_2O (pH 4.0; acetic acid), H_2O, and hexane to eliminate salts and unpolar lipids. Metabolites of AA were eluted with MeOH. Each eluate was evaporated under a nitrogen stream and reconstituted in 50 μl MeOH:H_2O (50:50). Eicosanoids were assayed by liquid chromatography and tandem mass spectrometry (LC-MS-MS), using a turbonebulizer-assisted electrospray (TurboIonSpray) interface coupled to a triple quadrupole MS (API-III; PE Sciex, Thornhill, Ont., Canada). Ten microliters of the samples were injected into the electrospray interface via the 20 μl loop of a Rheodyne injector (model 7125; Rheodyne, Cotati, Calif.) connected to a column (2×150 mm, packed with 5 μm $C_1$8 particles), using MeOH:H_2O (70:30, vol/vol, containing 0.01% acetic acid and 1 mM ammonium acetate) as solvent, at a flow rate of 200 μl/min. Samples were analyzed in the negative ion mode. The parent ion/daughter ion fragments monitored were 351/175 (PGE_2), 355/275 (PGE_2-D_4; internal standard), 369/169 (TXB_2), 369/245 (6-keto $PGF_{1\alpha}$), 351/233 (PGD_2), and 353/193 ($PGF_{2\alpha}$), respectively, each fragment being a specific marker for its respective parent molecule (25). Quantification of PGE_2 and TXB_2 in LC-MS-MS experiments was performed by comparing samples with the signal obtained from authentic PGE_2 and TXB_2 standards.

Measurement of PGE_2 by EIA

Evaluation of PGE_2 synthesis was performed as previously described (26), using a commercial enzyme immunoassay (EIA) kit (Cayman Chemical Co., Ann Arbor, Mich.). Cross-reactivities in the PGE_2 EIA were $<0.04\%$ for 6-keto $PGF_{1\alpha}$ and $<0.01\%$ for LTB_4, TXB_2, and AA.

Statistical analysis

Statistical analysis was performed by Student's unpaired t test (two-tailed), and significance was considered to be attained when P was < 0.05.

Reagents

Dextran T-500 and Ficoll-Paque were purchased from Pharmacia Fine Chemicals (Dorval, Québec). HBSS was from Gibco laboratories (Burlington, Ontario). Recombinant human interleukin 8 (IL-8) was a gift from Dr. Caroline Hébert (Genentech, South San Francisco, Calif.). Biosynthetic recombinant human GM-CSF was a gift of The Genetics Institute, Cambridge, Mass., and was dissolved in sterile buffered saline containing 0.01% human serum albumin; the stock solution (100 nM of GM-CSF) was stored at −80°C and handled aseptically. Biosynthetic recombinant human tumor necrosis factor α (TNF-α) with a specific activity of 10^7 U/mg was a generous gift from Knoll Pharmaceuticals (Whippany, N.J.). It was stored at −80°C in PBS containing 0.01% bovine serum albumin at a concentration of 10^6 U/ml. Tests on these solutions using the *Limulus* amoebocyte assay (Whittaker Bioproducts, Walkerville, Md.) for LPS were negative. LPS (*Escherichia coli* 0111-B4) was obtained from Difco Laboratories (Detroit, Mich.). The chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), the ionophore A23187, HEPES, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, Triton X-100, ponceau S, tween-20, propidium iodide, dimethyl sulfoxide, cycloheximide (CX), actinomycin D (AD), indomethacin, phorbol 12-myristate 13-acetate (PMA), herbimycin A, bovine serum albumin, dexamethasone (Dex), and PGB_2 were from Sigma Chemical Co. (St. Louis, Mo.). Cytochalasin B was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). The IgG_1 anti-CD14 monoclonal antibody (MEM18), which blocks binding of the LPS-LPS binding protein (LBP) complex to CD14, was a generous gift from Dr. Vaclav Horejsi (Laboratory of Leukocyte Antigens, Institute of Molecular Genetics, Videnska, PRAHA, Czech Republic). Its specificity was confirmed by International Workshops. [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole] (SB 203580) and [2- (4-methylsulfonyl)-3-[4-(2-methylpyridyl)]-6,7-dihydro [5H] pyrrolo [1, 2-α] imidazole] (SK&F 106978) were generously supplied by Dr. John C. Lee (SmithKline Beecham, King of Prussia, Pa.). All products for SDS-PAGE and protein blotting were of reagent grade and purchased from Pharmacia (Montreal, Québec). Rabbit polyclonal antibody was prepared against the peptide (C)-NASSSRSGLDDINPTVLLK, which is present only in the carboxyl-terminal of human COX-2 (19, 27). The specificity of this antibody toward the inducible isoform COX-2 has been extensively characterized previously

(24). Horseradish peroxidase-linked goat anti-rabbit purified antibody was purchased from BIO/CAN (Mississauga, Ontario). NS-398, PGE_2, and PGE_2-3, 3, 4, 4-d_4 were from Biomol Research Laboratories (Plymouth Meeting, Pa.). Leukotriene B_4, thromboxane B_2, and 6-keto-$PGF_{1\alpha}$ were purchased from Cayman Chemical Company (Ann Arbor, Mich.). All solvents were HPLC grade from Anachemia (Montreal, Québec). All other reagents were of analytical grade.

RESULTS

COX-2 protein and mRNA expression in human neutrophils

The ability of human neutrophils to express the mitogen-inducible cyclooxygenase isoform (COX-2) after activation of protein kinase C was assessed first. Cells were incubated for various periods of time with the phorbol ester PMA or its diluent and analyzed by immunoblot using an antibody that specifically recognizes the COX-2 isoform (24). Freshly isolated, unstimulated cells displayed no detectable COX-2. On the other hand, PMA time-dependently stimulated the appearance of a doublet with a relative molecular mass of 72–74 kDa. This doublet pattern is characteristic of the COX-2 isoform and likely represents differentially glycosylated forms of the enzyme (28) (**Fig. 1*A***). COX-2 could be detected after 30 min of incubation with PMA and the immunoreactive signal increased with time for up to 20 h. COX-2 was not detected at any of the time points in diluent-treated cells.

Selected phagocytic stimuli for the neutrophil were assessed for their capacity to induce COX-2. Cells were incubated for various periods of time with bacteria (B), serum-opsonized bacteria (Bop), zymosan (Z), or serum-opsonized zymosan (Zop). Although the two particles time-dependently induced the protein expression of COX-2 in neutrophils, their prior opsonization led to a more rapid and potent induction of the enzyme (Fig. 1*B*). Opsonized particles induced the protein expression of COX-2 within 30 min of incubation and the induction was sustained for up to 20 h, when the upper band of the doublet became prominent.

Time course incubations were also performed with neutrophil soluble agonists. Proinflammatory cytokines such as IL-1β and TNF-α, a bacterial wall byproduct, LPS, the chemotactic peptide fMLP, the growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF), and the C-X-C class chemokine IL-8 were assessed. As shown in Fig. 1*C*, TNF-α, LPS, GM-CSF, and fMLP all induced the expression of COX-2 in a time-dependent fashion. Preincubation with 10 μM cytochalasin B resulted in a more intense induction of COX-2 expression in fMLP-treated cells (Fig. 1*C*, inset). The most rapid response within this group of agonists was obtained with fMLP, and immunoreactive COX-2 could be detected as early as 30 min after stimulation. The levels of the enzyme increased with the length of incubation [up to 1 h(fMLP) or 4 h (LPS, GM-CSF, and TNF-α)], and the enzyme was still detectable after 20 h. At that time, the upper band appeared to be prominent, in some cases being the only remaining immunoreactive band. IL-8 did not lead to a detectable expression of COX-2. IL-1β also failed to induce any detectable levels of COX-2 in the neutrophil. COX-1 protein was barely detectable in unstimulated neutrophil preparations as assessed by immunoblotting and no significant change of immunoreactivity could be observed after cell treatment (data not shown). As determined by densitometric analysis of the doublets after 4 h of incubation in these experiments, PMA triggered the greatest COX-2 induction with an approximately 8.5-fold increase of the immunoreactive doublet. Zop and TNF-α were also potent stimuli of COX-2 induction, closely followed by GM-CSF, LPS, fMLP, and Bop (Fig. 1*D*).

The involvement of transcriptional and translational events for agonist-triggered COX-2 protein expression in the neutrophil was addressed. Cells were incubated for 1 h *1)* with either PMA, fMLP, LPS, Zop, TNF-α, or GM-CSF alone, *2)* in combination with the transcription inhibitor actinomycin D (AD), or *3)* with the protein synthesis inhibitor CX. Similar results were obtained with every agonist; blocking transcription with AD only partially blocked the stimulation of COX-2 protein expression, whereas inhibition of translation by CX consistently decreased COX-2 expression below the limits of detection (**Fig. 2**).

Total RNA from human neutrophils was extracted in order to evaluate the expression of COX-2 mRNA by Northern blot. In these experiments, freshly isolated, unstimulated cells displayed low but detectable levels of COX-2 mRNA. Incubation of the cells with PMA induced a time-dependent increase in the expression of COX-2 mRNA. A band of approximately 4 kb along with two secondary bands became visible after 30 min of incubation with PMA; this increase was sustained until the 60 min time point. In unstimulated cells, the COX-2 mRNA levels were present but relatively low at each time point (**Fig. 3*A***). Incubation of the cells for 60 min with fMLP, TNF-α, GM-CSF, Zop, or LPS also led to significant increases in COX-2 mRNA levels compared to untreated cells. At that time, the levels of COX-2 mRNA were highest in fMLP, TNF-α, and LPS-stimulated neutrophils, and GM-CSF led to a weaker increase. On the other hand, incubation with IL-1β did not significantly alter COX-2 mRNA levels (Fig. 3*B*).

Regulation of COX-2 expression in human neutrophils

Experiments were performed to assess the effect of serum and the involvement of the CD14 surface an-

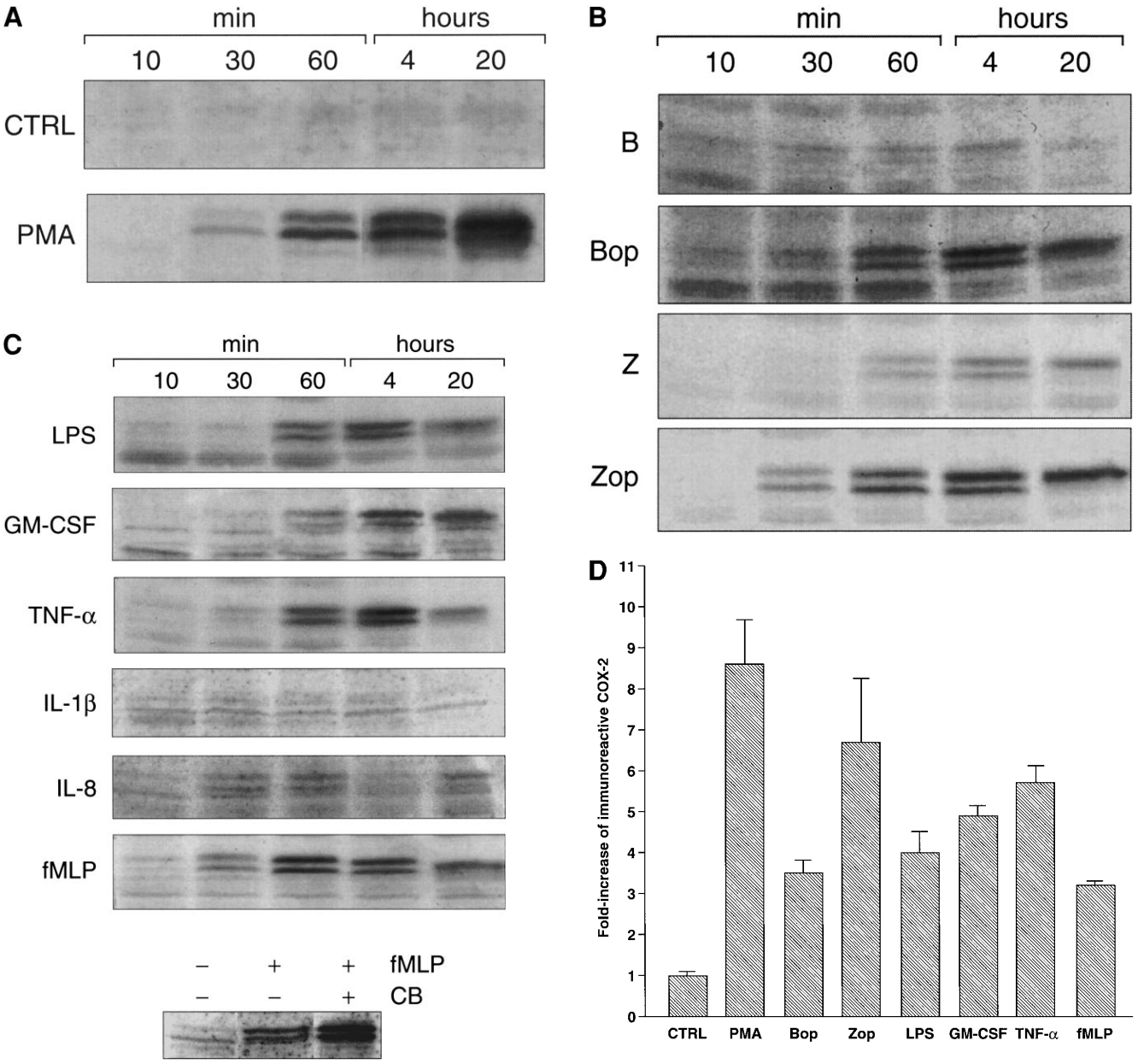

Figure 1. Stimulation of COX-2 expression in human neutrophils by different classes of agonists. Neutrophils were stimulated and processed for determination of COX-2 expression by immunoblotting, as described in Materials and Methods. Immunoblots are representative of at least three independent experiments performed with different donors under identical conditions. *A)* Effect of PMA on the expression of COX-2. Neutrophils were incubated for the times indicated in the absence or presence of 100 nM PMA. *B)* Effect of phagocytic stimuli on the expression of COX-2. Neutrophils were incubated in the presence of bacteria (B; 10^8 bacteria/ml), opsonized bacteria (Bop; 10^8 bacteria/ml), zymosan (Z; 300 μg/ml), or opsonized zymosan (Zop; 300 μg/ml). *C)* Effect of soluble agonists on the expression of COX-2. Neutrophils were incubated with LPS (1 μg/ml in the presence of 1% FCS), GM-CSF (1 nM), TNF-α (100 nM), IL-1β (100 nM), IL-8 (100 nM), or fMLP (100 nM). Inset: Neutrophils were incubated for 60 min with fMLP in the absence (−) or presence (+) of 10 μM cytochalasin B (CB). *D)* Densitometric measurement of COX-2 expression induced by different neutrophil agonists. Agonist-induced COX-2 expression was evaluated by densitometric analysis of the 4 h time point, as described in Materials and Methods. Results are presented in fold increase of densitometric measurements from the 10 min to the 4 h time point (mean±SEM, n=3).

tigen on the induction of COX-2 protein expression by LPS. Neutrophils were incubated with various concentrations of LPS for 60 min in the absence or presence of 1% FCS. The capacity of LPS to induce COX-2 in neutrophils was potentiated by approximately 100-fold by serum. (**Fig. 4*A***). A protein present in the plasma (LBP) forms a complex with LPS, which may interact with neutrophils through the CD14 surface marker. In our experiments, the presence of a monoclonal antibody (MEM18) that specifically blocks the interaction between the LPS-LBP complex and CD14 effectively prevented the induction of COX-2 by LPS

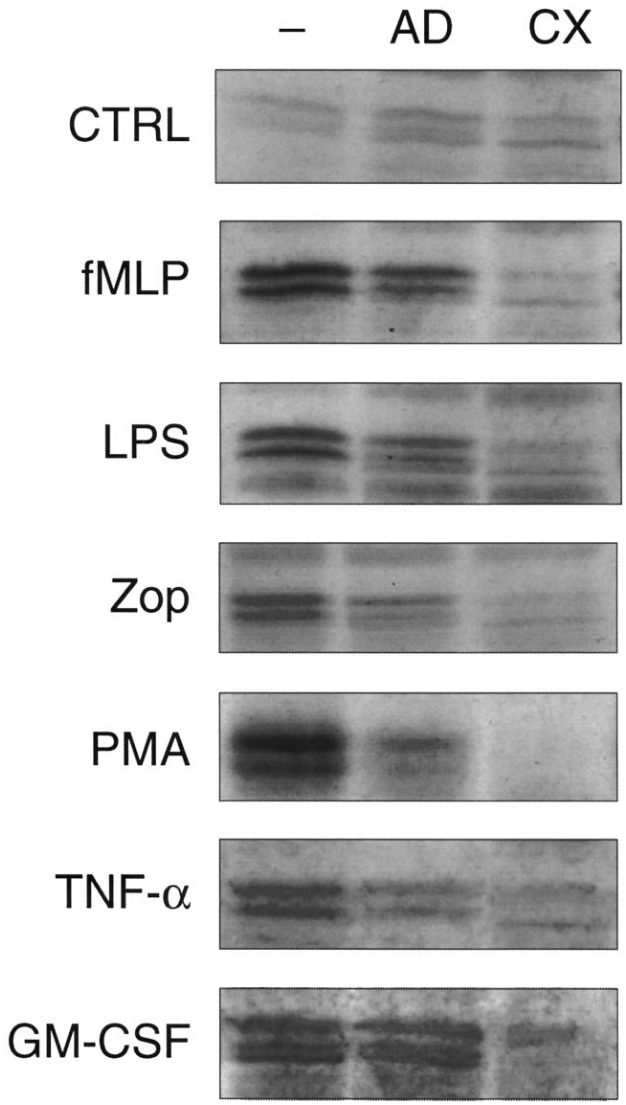

Figure 2. Effect of transcription and protein synthesis inhibitors on COX-2 expression in human neutrophils. Neutrophils were incubated for 1 h in HBSS or with the indicated agonists, alone or in combination with the transcription inhibitor actinomycin D (AD; 5 μg/ml) or the protein synthesis inhibitor cycloheximide (CX; 20 μg/ml), and processed for determination of COX-2 expression by immunoblotting. When LPS was used as an agonist, incubations were performed in the presence of 1% FCS. For each agonist, an immunoblot representative of at least three independent experiments is shown.

(Fig. 4*B*). An isotype-matched irrelevant antibody (6F4B6) was without effect.

Activation of p38 MAPK is required for optimal COX-2 induction in stimulated human monocytes, fibroblasts, and vascular endothelial cells, as prevention of p38 MAPK activation by the specific inhibitor SB 203580 effectively blocks the induction of COX-2 in these cells (29, 30). We therefore investigated the potential role of p38 MAPK on the expression of COX-2 in neutrophils. In contrast to previous observations in other cell types, SB 203580 had no discernible effect on the expression of COX-2 in neutrophils incubated with different classes of agonists (**Fig. 5*A***).

We sought to determine the effect of a synthetic glucocorticoid, Dex, on the stimulated expression of COX-2 in human neutrophils. Cells were incubated with different classes of agonists in the presence of diluent or Dex at concentrations ranging from 10 pM to 1 μM. Under these experimental conditions, Dex showed a weak inhibitory effect on the stimulated expression of COX-2 only at the highest concentration used. A typical immunoblot of fMLP-treated neutrophils is presented in Fig. 5*B*. Similar results were obtained with the other agonists inasmuch as no significant effect of Dex on COX-2 protein expression was observed at concentrations below 1 μM (data not shown).

Phospholipase D (PLD), which hydrolyzes phospholipids (primarily phosphatidylcholine) to generate phosphatidic acid (PA), has emerged as a critical component in cellular signal transduction. PA and its

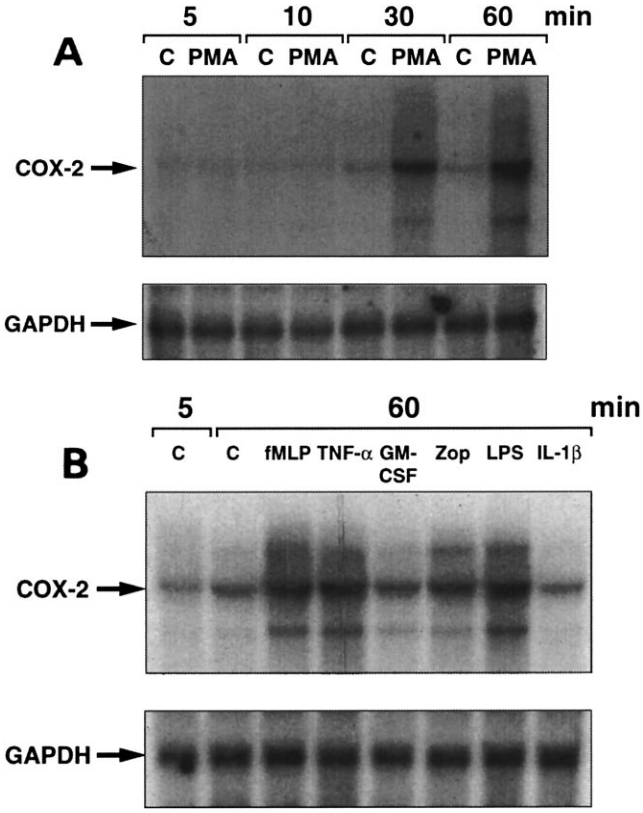

Figure 3. Effects of agonists on COX-2 mRNA levels in human neutrophils. Cells were incubated under the conditions indicated and samples were processed for evaluation of COX-2 mRNA levels by Northern blotting, as described in Materials and Methods. *A)* Kinetics of COX-2 mRNA levels in PMA-stimulated neutrophils. Neutrophils were incubated with or without PMA for the indicated times. *B)* Effect of various agonists on the levels of COX-2 mRNA. Neutrophils were stimulated for 30 min in the presence of the indicated agonists. In each experiment, filters were hybridized with GAPDH cDNA to ensure equal RNA loading and integrity.

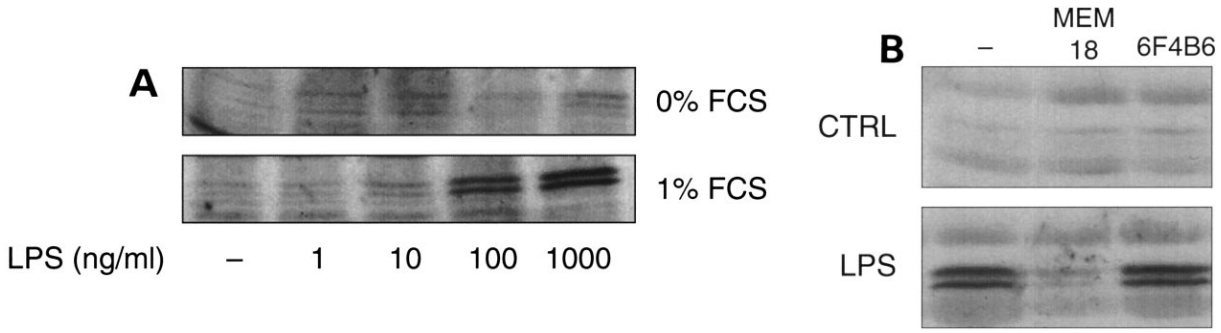

Figure 4. Assessment of the involvement of the CD14 antigen in LPS-induced COX-2 protein expression in neutrophils. *A)* Effect of serum on induction by LPS of COX-2. Neutrophils were incubated for 1 h with increasing concentrations of LPS in the absence or presence of 1% FCS, and samples were processed for determination of COX-2 expression by immunoblotting. *B)* Effect of an anti-CD14 monoclonal antibody on COX-2 induction by LPS. Neutrophils were incubated for 1 h with 1 μg/ml LPS alone or in combination with a monoclonal anti-CD14 blocking antibody (MEM18) or a matched-type monoclonal antibody (IgG_1; 6F4B6) and processed for immunoblotting. Immunoblots representative of three separate experiments are shown.

dephosphorylated product, sn-1,2-diacylglycerol, are important intracellular second messengers. The coupling of PLD to specific receptors occurs through multiple mechanisms involving protein kinase C, protein tyrosine kinase, Ca^{2+}, and GTP binding proteins. The presence of ethanol (EtOH) prevents the production of second-messenger phosphatidic acid, and leads instead to the production of phosphatidylethanol (31). We incubated the neutrophils with concentrations of EtOH ranging from 0.1 to 2.0% (v/v) in order to assess the potential involvement of PLD activation in the induction of COX-2. EtOH inhibited the PMA-elicited expression of COX-2 in a concentration-dependent fashion. Since butanol has been shown to be a better transphosphatidylation substrate for PLD than EtOH (32), we sought to determine whether the capacity of primary alcohols to inhibit neutrophil COX-2 expression was related to their ability to serve as transphosphatidylation substrates and thereby as an inhibitor of phosphatidic acid formation in stimulated neutrophils. Butanol was more potent than EtOH in inhibiting COX-2 induction (**Fig. 6*A***), in agreement with its better ability to serve as a transphosphatidylation substrate for PLD. The effect of 1.0% EtOH on the protein expression of COX-2 was evaluated in cells treated with different agonists including PMA, LPS, Zop, fMLP, and TNF-α. The results of these experiments indicate that EtOH has differential effects on COX-2 protein expression, depending on the agonist used (Fig. 6*B*). As evaluated by densitometric analysis of immunoreactive COX-2 in three separate experiments, inductions by LPS or by TNF-α were only minimally affected by the presence of EtOH. PMA- and fMLP-triggered COX-2 protein expression was decreased by approximately 50%. The induction triggered by Zop was that most affected by EtOH, being decreased by approximately 75% (Fig. 6*C*). The activation of PLD therefore seems to be variably involved in the induction of COX-2, depending on which agonist is used.

Identification of COX-derived metabolites in agonist-activated neutrophils

Cells were incubated for 20 h with a selection of agonists capable of inducing COX-2 protein expression and processed for the identification of COX-derived

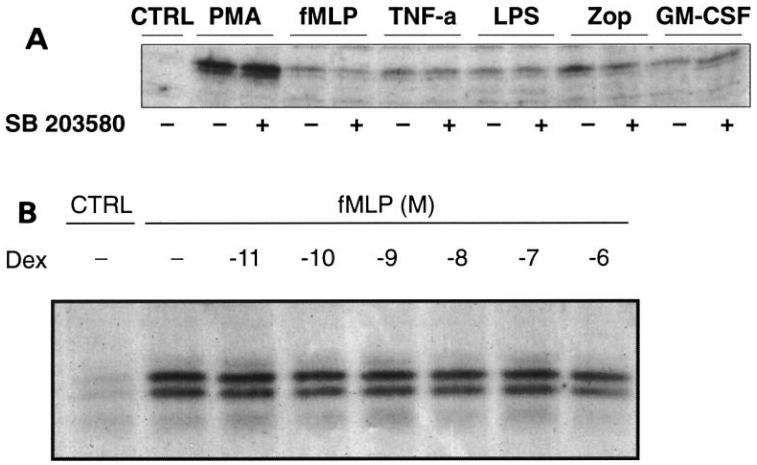

Figure 5. Effect of selected pharmacological agents on COX-2 expression in neutrophils. Cells were incubated for 60 min with the indicated agonists and agents, and samples were processed for evaluation of COX-2 protein expression by immunoblotting. *A)* Effect of a specific p38 MAPK inhibitor on the stimulated protein expression of COX-2. Cells were incubated in the absence or presence of 1 μM SB 203580. A blot representative of four separate experiments is shown. *B)* Effect of a synthetic glucocorticoid on the stimulated protein expression of COX-2. Cells were incubated in the absence or presence of dexamethasone (Dex). An immunoblot representative of two separate experiments is shown.

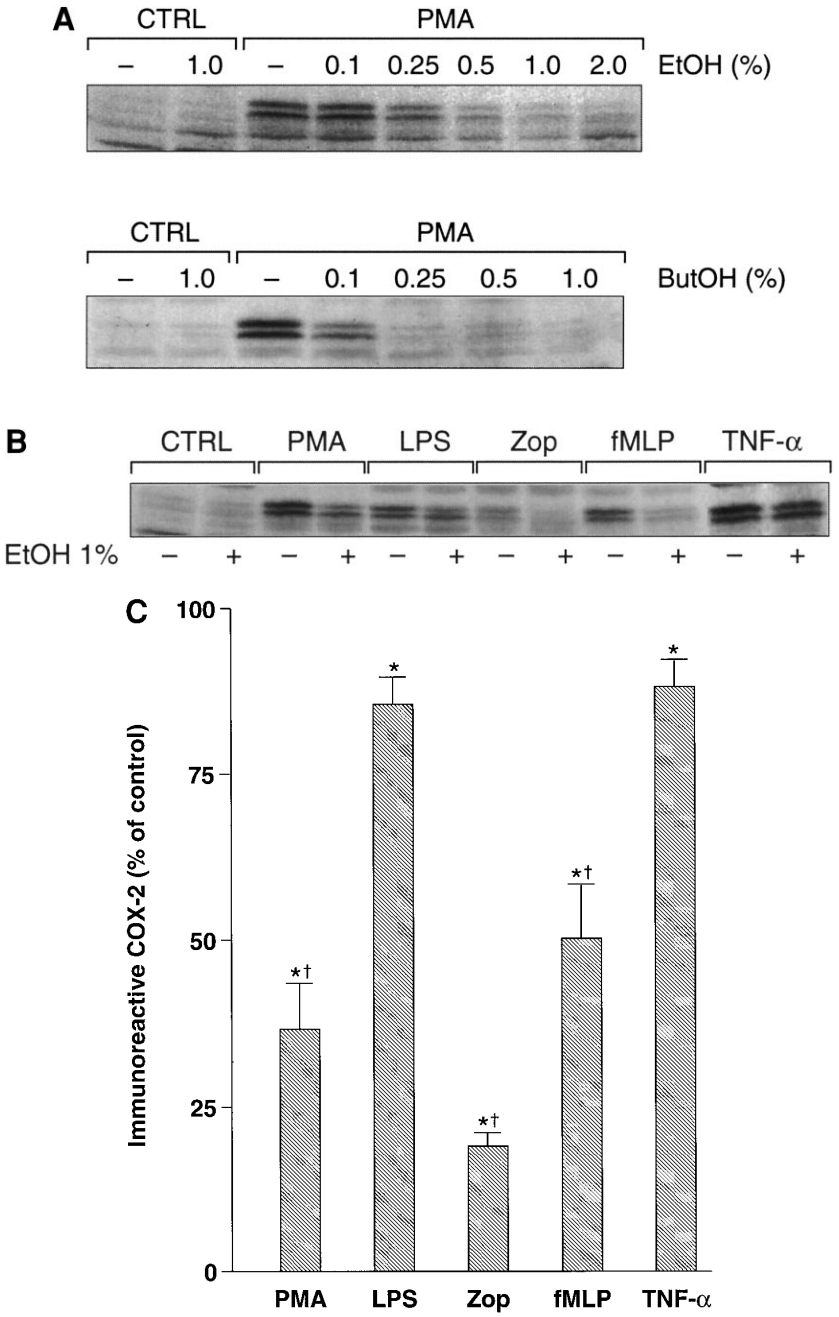

Figure 6. Effect of ethanol and butanol on the expression of COX-2 in neutrophils. Cells were incubated for 60 min and samples were processed for evaluation of COX-2 protein levels by immunoblotting. *A)* Effect of ethanol and butanol on PMA-triggered COX-2 protein expression. Cells were stimulated with PMA alone or in the presence of increasing concentrations of ethanol (EtOH) or butanol (ButOH). *B)* Effect of ethanol on COX-2 protein expression in neutrophils treated with different inflammatory agents. Cells were stimulated with agonists alone or in the presence of 1% ethanol. Typical immunoblots, representative of at least three separate experiments, are shown. *C)* Densitometric analysis of the effects of ethanol on the stimulated expression of COX-2. Levels of immunoreactive COX-2 were evaluated by densitometric analysis of the doublets. Results are expressed as percent (mean±SEM, n=3) of control (COX-2 levels in cells stimulated in the absence of EtOH). *Significantly different from control; †significantly different from LPS or TNF-α stimulated cells.

metabolites by LC-MS-MS, as described in Materials and Methods. In each experiment, samples were analyzed for the presence of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, the latter two being evaluated by monitoring their stable derivatives 6-keto-$PGF_{1\alpha}$ and TXB_2, respectively. Resting cells did not yield detectable amounts of these eicosanoids. Incubation with PMA, on the other hand, led to the production of TXB_2 and PGE_2 (**Fig. 7*A***). The COX-2 inhibitor NS-398 (1 μM) effectively prevented PMA-stimulated TXB_2 and PGE_2 synthesis by 89 ± 5% and 73 ± 2%, respectively (mean±SEM; n=4). The nonspecific COX inhibitor

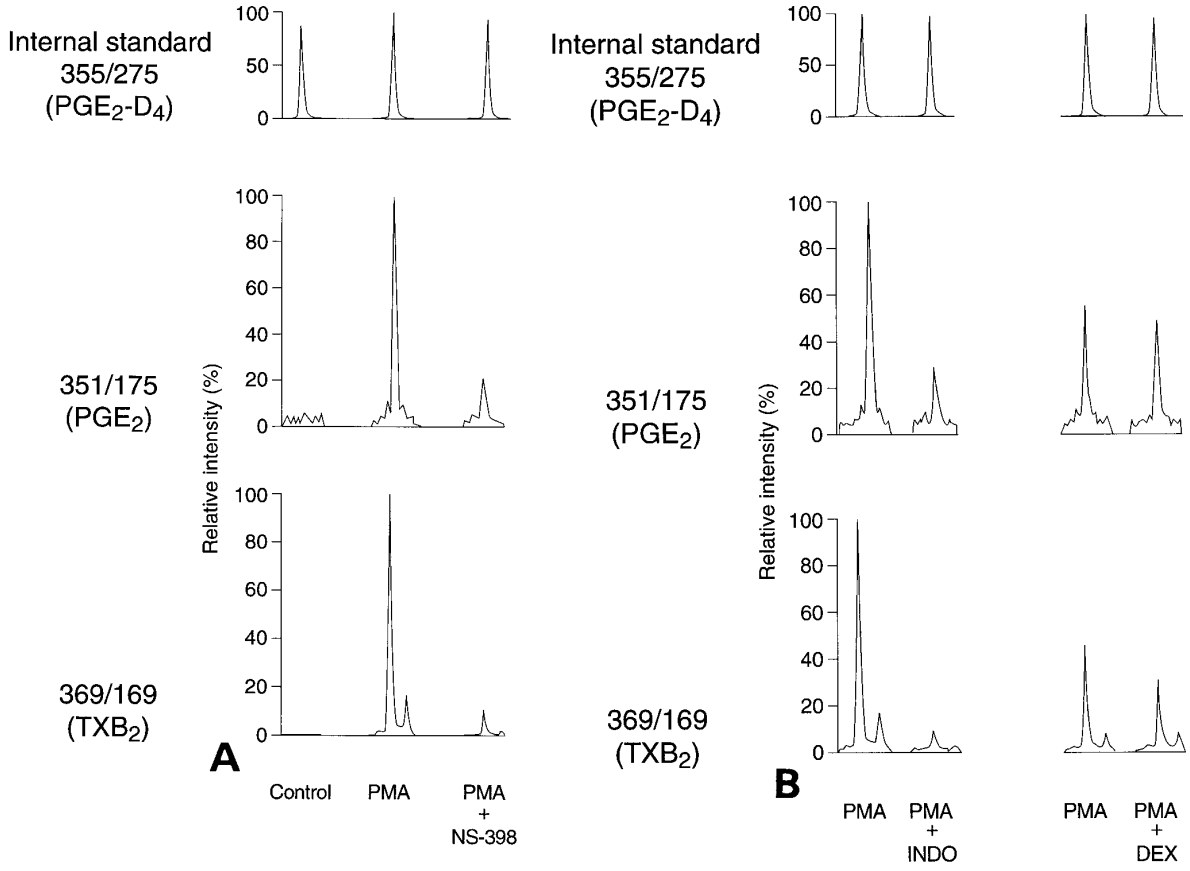

Figure 7. LC-MS-MS analysis of cyclooxygenase-derived products from stimulated human neutrophils. Cells were treated for 20 h with PMA and samples were processed for the idenfication of COX-derived metabolites by LC-MS-MS, as described in Materials and Methods. Samples were spiked with deuterium-labeled PGE_2-D_4, which was used as an internal standard. Reactions accounting for the presence of PGD_2, PGE_2, $PGF_{2\alpha}$, and the stable metabolites of TXA_2, PGI_2, TXB_2, and 6-keto $PGF_{1\alpha}$, respectively, were monitored. *A)* Effect of the specific COX-2 inhibitor, NS-398, on PMA-triggered COX product synthesis. Cells were treated for 20 h with PMA, alone or in the presence of NS-398 (1 μM). *B)* Effect of the COX inhibitor indomethacin and of dexamethasone on PMA-triggered COX product synthesis. Cells were stimulated for 20 h with PMA, alone or in the presence of Indo (1 μM) or dexamethasone (Dex; 1 μM). Data for PGE_2-D_4, PGE_2, and TXB_2 are presented; PGD_2, $PGF_{2\alpha}$, or 6-keto $PGF_{1\alpha}$ were not detected. Results are from one experiment representative of at least two other experiments.

indomethacin (Indo; 1 μM) blocked the synthesis of both eicosanoids to an extent similar to that of NS-398, inhibiting PMA-triggered synthesis of TXB_2 and PGE_2 by 92 ± 3% and 70 ± 6%, respectively (mean±SEM; n=2). The presence of Dex (1 μM) had only a marginal effect on eicosanoid production in PMA-treated cells, diminishing TXB_2 and PGE_2 synthesis by 24 ± 5% and 5 ± 3% (mean±SEM; n=3), respectively (Fig. 7*B*). Other agonists (Zop, TNF-α, and fMLP) led to qualitatively similar results, inasmuch as TXB_2 and PGE_2 were the only COX-derived metabolites detected and their synthesis was significantly prevented by NS-398 (data not shown). In all experiments, TXB_2 was produced in greater amounts than PGE_2; however, whereas the treatment of the cells with PMA produced a TXB_2/PGE_2 molar ratio of 2.7 ± 0.1 (mean±SEM; n=6), the ratio was 1.5 ± 0.1 (n=3) for all other agonists. Other COX-derived metabolites, including PGD_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$, were looked for and consistently not detected under all conditions used. These results identify TXB_2 and PGE_2 as the main COX-derived metabolites produced by activated human neutrophils. They also confirm the capacity of neutrophils to synthesize eicosanoids from endogenous AA via the COX-2 pathway.

PGE_2 synthesis by neutrophils treated with selected agonists was also measured by EIA, as described in Materials and Methods. In time course experiments, PGE_2 was first detected after 4 h of incubation with PMA, and levels of PGE_2 continued to increase for up to 20 h. Incubation with other agonists yielded similar kinetics, albeit with different magnitudes (**Fig. 8*A***). Among the agonists used, PMA was the most potent and elicited synthesis of approximately 9 ng of PGE_2 after 20 h of incubation. PMA was followed by (in decreasing order) Bop, Zop, Z, TNF-α, and fMLP (Fig. 8*B*). Despite their ability to induce COX-2 in a commensurable way, LPS and GM-CSF did not trigger any detectable synthesis of PGE_2 under these experimental conditions.

Inhibition of protein synthesis by CX effectively blocked PGE_2 synthesis in response to all classes of

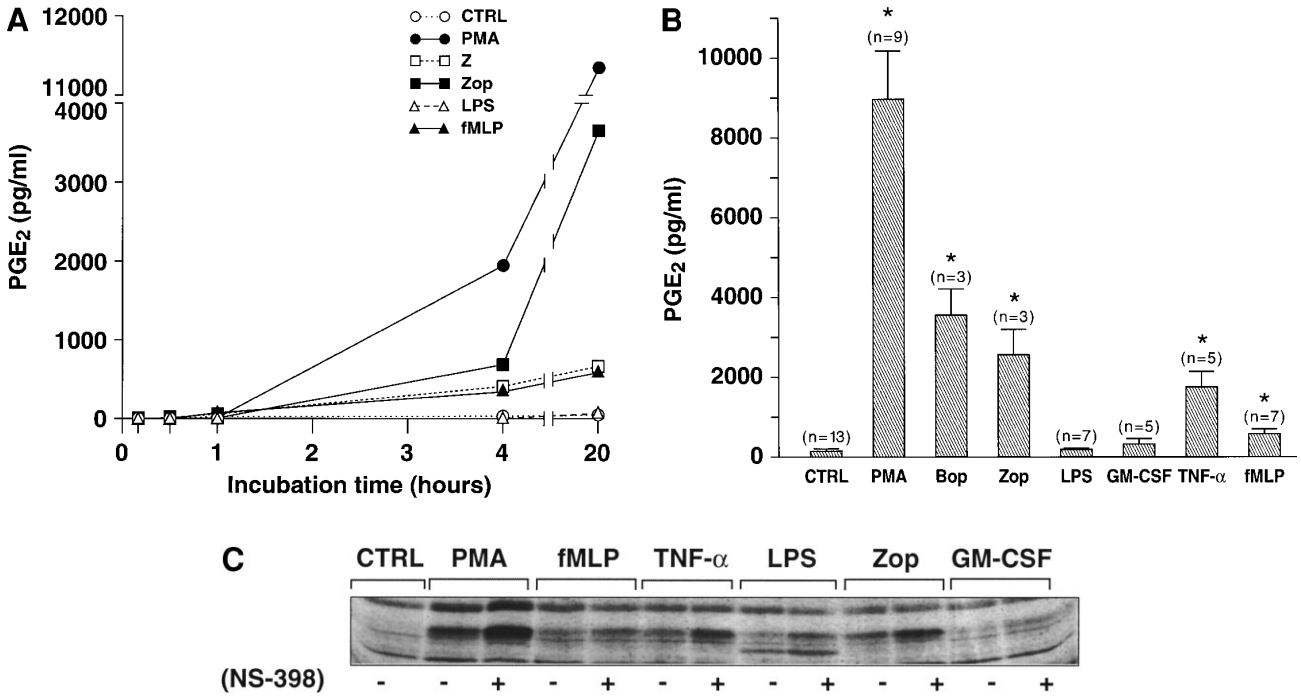

Figure 8. Characteristics of the PGE_2 synthesis by stimulated neutrophils. Neutrophils (10×10^6/ml) were incubated as indicated and incubation media were processed for PGE_2 quantitation by EIA as described in Materials and Methods. *A)* Time course of PGE_2 synthesis by activated neutrophils. Cells were incubated for increasing periods of time with the indicated agonists. Results, presented in pg/ml, are from one experiment representative of two others. *B)* PGE_2 synthesis by neutrophils stimulated by various inflammatory agents. Cells were stimulated for 20 h with the indicated agonists. Results are presented in pg/ml and are the mean (±SEM) of at least three separate experiments. *Significantly different from control cells. *C)* Effect of NS-398 on the COX-2 protein expression in activated neutrophils. Neutrophils were stimulated for 20 h with the indicated agonists and samples were processed for evaluation of COX-2 protein levels by immunoblotting. An immunoblot representative of three separate experiments is shown.

agonists. NS-398 also blocked the synthesis of PGE_2 in all conditions tested (**Table 1**). The latter inhibition by NS-398 was not due to a decrease in COX-2 protein synthesis, as NS-398 actually caused an increase of the immunoreactive levels of COX-2 protein in neutrophils incubated with different agonists (Fig. 8*C*). In addition to confirming the data obtained by LC-MS-MS, the present data further substantiate a preeminent role of COX-2 for eicosanoid synthesis by the neutrophil.

COX-2 expression, substrate availability, and PGE_2 synthesis by human neutrophils

LPS and GM-CSF induced COX-2 protein expression in neutrophils, but did not trigger any significant PGE_2 synthesis when compared to untreated cells. Experiments were conducted to determine whether stimulation of endogenous AA release could result in enhanced PGE_2 synthesis by the neutrophil's COX-2. In this set of experiments, COX-2 protein expression was induced by incubating the cells with LPS for 2 h. Neutrophils were then challenged with the divalent cation ionophore A23187 (1 μM) for 5 min, and reactions were stopped by rapid cooling of the samples. PGE_2 synthesis was evaluated by EIA. Incubation with LPS alone did not induce significant PGE_2 synthesis when compared to control cells (incubated in the absence of LPS). Stimulation of the control cells with A23187 caused a modest production of PGE_2. In contrast, stimulation with A23187 strikingly enhanced

TABLE 1. *Inhibition by metabolic inhibitors of stimulated-PGE_2 synthesis*[a]

	PMA	Zop	TNF-α	fMLP
Cycloheximide	91 ± 3 (n = 2)	73 ± 6 (n = 2)	97 ± 2 (n = 2)	74 ± 10 (n = 2)
NS-398	86 ± 3 (n = 7)	87 ± 5 (n = 2)	87 ± 2 (n = 4)	87 ± 7 (n = 4)

[a] Neutrophils were stimulated for 20 h with the indicated agonists alone or in the presence of 20 μg/ml cycloheximide or 1 μM NS-398. PGE_2 synthesis was then determined by EIA, as described in Materials and Methods. Results shown are the percentage of inhibition by the metabolic inhibitor (mean±SEM) when compared to the PGE_2 synthesis from stimulated cells in the absence of inhibitor.

the production of PGE_2 by LPS-incubated cells (**Fig. 9**). A similar set of experiments was performed using physiological agonists instead of A23187. After exposure to LPS, cells were challenged with 100 nM fMLP or 300 nM platelet-activating factor (PAF). Stimulation with either fMLP or PAF of LPS-treated cells resulted in a significant increase of PGE_2 synthesis. In the presence of NS-398, this stimulated-PGE_2 synthesis was inhibited by approximately 95% (data not shown).

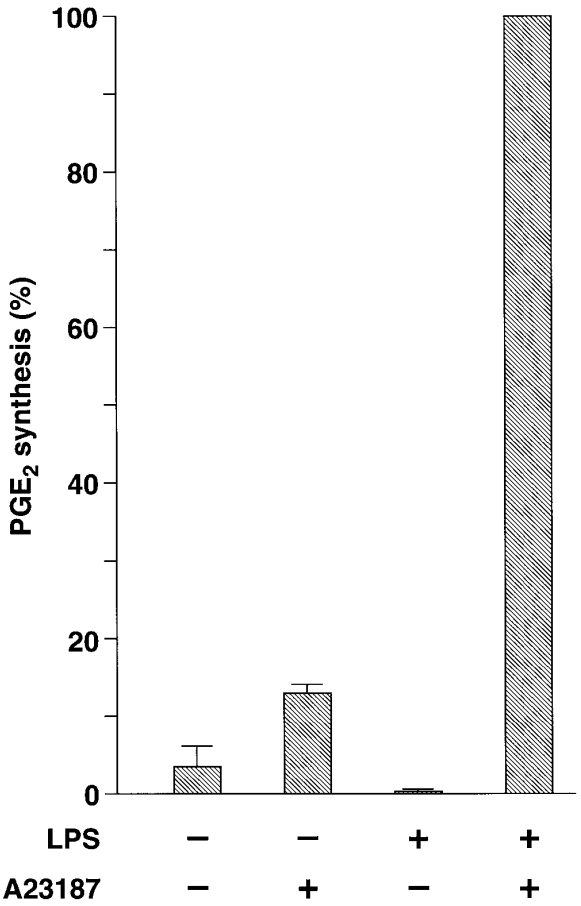

Figure 9. Involvement of AA availability and COX-2 expression for the synthesis of PGE_2 by human neutrophils. Cells were incubated for 2 h in the absence or presence of 1 μg/ml LPS, then challenged with the divalent cation ionophore A23187 (1 μM) or an equal volume of HBSS for 5 min. PGE_2 synthesis was then determined by EIA. Cells incubated with LPS and stimulated with A23187 resulted in the synthesis of 2.7 ± 0.7 ng (mean±SEM, n=4). Results are expressed as the percentage of PGE_2 synthesis in each sample compared to that from cells incubated with LPS and stimulated with A23187; they represent the mean ± SEM from four distinct experiments conducted under identical conditions.

DISCUSSION

A number of inflammatory factors were identified that up-regulate the expression of COX-2 in human neutrophils. First, activation of PKC by PMA potently induced COX-2. Second, inflammatory stimuli, including Zop and Bop, LPS, GM-CSF, TNF-α, and fMLP, increased the expression of this early-response gene. The COX-2 induction by these agents was rapid, especially in the case of fMLP, where de novo synthesized enzyme was detected within 30 min of exposure to the peptide. In comparison to the other agents tested, the C-X-C chemokine IL-8 was a weak COX-2 inducer. IL-1β failed to stimulate COX-2 expression in neutrophils under the experimental conditions used in this study. In several cell types, including human monocytes (33), synovial cells (34, 35), dermal fibroblasts (36), astrocytes (37), amnion-derived wish cells (38), chondrocytes (39), smooth muscle cells (40), pulmonary epithelial cells (41), and several other types (42–44), IL-1β caused the induction of COX-2. The only circulating cell type (other than neutrophils) in which COX-2 has been shown to be expressed is the monocyte. The differential responsiveness to IL-1β in these two cell types highlights the distinct roles that neutrophils and monocytes may play in the inflammatory response.

It has previously been shown that the ability of LPS to prime isolated neutrophils for enhanced functional responses is dependent on the presence of plasma and involves a CD14-dependent mechanism (45). Results from the present study confirm these observations and extend the involvement of LBP and CD14 to the LPS-elicited COX-2 expression in neutrophils. Different classes of inflammatory agents interacting with receptors that belong to different families, including receptors linked to heterotrimeric GTP binding proteins (fMLP) or to tyrosine kinases (GM-CSF), the $F_{C\gamma}$ receptors (opsonized particles, LPS), and the ceramide-linked receptors (TNF-α), induced COX-2 expression. Each class of receptor may then activate specific and distinct signal transduction cascades, which may then converge toward a common path leading to up-regulation of the COX-2 gene. In addition, some of the agents tested stimulated aggregation, degranulation, and superoxide production by the neutrophil. It is therefore possible that the effects of some of these agents could have been mediated indirectly by some of the released neutrophil-derived products.

For many agonists, downstream events that follow their binding to neutrophil receptors remain unclear. However, the stimulation of p38 MAPK has been positively linked to the induction of COX-2 in human monocytes, fibroblasts, and vascular endothelial cells (29, 30). We therefore sought to determine the effect of a specific p38 MAPK inhibitor, SB 203580, on the stimulated protein expression of COX-2 in human neutrophils. When used at a concentration that is effective for blocking the expression of COX-2 in monocytes stimulated with different agonists (29), SB 203580 had no significant effect on the expression of COX-2 in the present study. MAPK

cascade has been recognized as a major signaling pathway transmitting signals from growth factors, hormones, and cytokines to the early-response genes (46); it is possible that other MAPKs, such as ERK-1 and ERK-2 or c-jun amino-terminal kinases/stress-activated protein kinases (JNK/SAPKs), may be involved in this response instead of p38 MAPK. The present data nevertheless suggest that the p38 MAPK-mediated pathway is not central to COX-2 gene expression in neutrophils.

At least part of the anti-inflammatory action of glucocorticoids is due to the suppression of COX-2 induction by proinflammatory stimuli (47). However, the induction of COX-2 in neutrophils was relatively insensitive to Dex treatment. Concentrations of the glucocorticoid that were effective for up-regulating 5-lipoxygenase-activating protein (FLAP) in human neutrophils (4) did not modify the expression of COX-2. Accordingly, when used at 1 μM, Dex had only a marginal inhibitory effect on PMA-triggered PGE_2 and TXA_2 synthesis. This contrasts with other cell types, such as monocytes/macrophages, fibroblasts, endothelial cells, and related cell lines, where glucocorticoids block the induction of COX-2 and inhibit PG synthesis (15, 20, 42, 47, 48). In certain cells, however, corticosteroids act paradoxically by stimulating rather than inhibiting PG production. Cultures of human amnion cells increase their capacity to convert exogenous AA to PGE_2 in response to glucocorticoid treatment (49, 50). A recent study also reported that Dex treatment of amnion cells resulted in an increase of COX-2 mRNA levels (51). These results, along with the lack of effects of IL-1β and the insensitivity to the p38 MAPK inhibitor, suggest that the expression of COX-2 in neutrophils relies on mechanisms that significantly differ from those previously implicated in other human circulating cells, namely, monocytes. They also demonstrate that the COX-2 signals (protein and mRNA) monitored in the present study do not originate from contaminating monocytes.

Transcription and translation are necessary for the induction of COX-2 in human neutrophils. However, inhibition of transcription only partially blocked COX-2 induction, suggesting that COX-2 mRNA may be constitutively expressed in neutrophils. Although COX-2 mRNA was indeed detected in fresh unstimulated cells, it remains to be established whether this is the result of a constitutive expression or is a consequence of the cell isolation procedures. Indeed, the latter may induce partial cell activation, which in turn results in an up-regulation of COX-2 mRNA expression. The human COX-2 gene produces two major transcript isoforms, 4.6 and 2.8 kb in length, which originate from alternative polyadenylation in the 3′-untranslated region (52). In the present study, the 4.6 kb form was prominent over the 2.8 kb one under all conditions assessed. An additional and relatively weak band of approximately 6 kb was sometimes observed; the identity of this band is currently unknown. The levels of COX-2 mRNA rapidly increased after stimulation by several inflammatory agents, suggesting that the up-regulation of COX-2 mRNA is an integral part of the stimulation of COX-2 enzyme expression by these agents. Accordingly, the 5′-flanking regions of COX-2 genes from different species, including humans, are typical of early-response genes and contain a number of *cis*-acting elements such as CRE, AP2, Sp1, and NF-IL6 (53–57) that can participate in the transcriptional regulation of the COX-2 gene (55, 56, 58). As underscored by the complexity of the regulation of the COX-2 gene in multiple tissues, additional studies will be required to identify elements that are involved in the transcriptional expression of this gene in neutrophils.

Inhibition of PLD by the addition of primary alcohols reduced the stimulated expression of COX-2 in neutrophils. The capacity of EtOH and ButOH to inhibit neutrophil COX-2 expression correlated with their ability to serve as transphosphatidylation substrates and inhibitors of phosphatidic acid formation. The effect of alcohols was concentration dependent and agonist specific. Only the responses to agonists that have previously been shown to stimulate PLD activity in human neutrophils (PMA, Zop, and fMLP but not LPS or TNF-α) were significantly inhibited by EtOH (59, 60). Although TNF-α has been shown to prime neutrophils and to enhance PLD activity in response to fMLP or IL-8, it does not by itself affect PLD activity (61, 62). Until now, no stimulation of PLD activity by LPS has been reported in neutrophils. These results suggest that distinct PLD-dependent and PLD-independent signaling pathways regulate the expression of COX-2 in neutrophils. PLD-derived products have recently been reported to be involved in the induction of COX-2 by phorbol esters in kidney cells (63). However, it is unclear whether the effects are due to phosphatidic acid itself or to phosphatidic acid-derived diacylglycerol. In T lymphocytes, PLD stimulation activates the transcription factor AP-1 in a PKC-dependent manner (64). Many proteins are involved in the regulation of transcription factors; their phosphorylation by phosphatidic acid or diacylglycerol-activated kinases could provide the link between PLD activation and RNA/protein synthesis. Additional studies will be required to identify the signaling proteins involved in the transcriptional expression of COX-2 in neutrophils.

Whereas several of the stimuli tested increased the levels of immunoreactive COX-2 to similar levels, the amplitude of prostanoid synthesis differed significantly depending on the agonist. For example, fMLP significantly increased COX-2 levels but was a relatively weak stimulus for the synthesis of PGE_2. In addition, the stimulation by GM-CSF or LPS consistently

failed to stimulate PGE_2 synthesis. The latter result contrasts with one from an earlier study where LPS alone triggered the synthesis of PGE_2 from neutrophils (22). This apparent discrepancy can be related to the concentrations of serum used, which were 10-fold higher than in the present report. The presence of cytokines, growth factors, and other agents in the serum may have potentiated the effect of LPS. These results also underlined the requirement of AA for the production of prostanoids by COX-2 in inflammatory neutrophils. Indeed, in LPS-treated cells, A23187 led to the production of significant amounts of PGE_2. Moreover, PGE_2 synthesis from LPS-treated neutrophils could also be triggered after stimulation by physiological agonists known to induce the release of AA such as fMLP or PAF. These results demonstrate that the LPS-induced COX-2 is present in an active state and that endogenous AA released upon further stimulation of inflammatory neutrophils by agonists is readily used by COX-2 for synthesis of PGE_2.

It was important to determine which cyclooxygenase isoform was responsible for the stimulated synthesis of eicosanoids in our studies. The specific COX-2 inhibitor NS-398 effectively blocked the synthesis of PGE_2 and TXA_2 induced by all agonists that triggered the expression of COX-2 protein. Furthermore, inhibition of protein synthesis prevented the synthesis of eicosanoids, thus confirming that the inducible COX isoform is primarily involved in eicosanoid synthesis by activated human neutrophils. Whereas TXA_2 is considered a proinflammatory compound, PGE_2 has been shown to exert both pro- and anti-inflammatory actions. The effect that the enhanced synthesis of these two eicosanoids may have on neutrophil functions in inflammatory situations therefore remains to be established; the relative abundance of bioactive eicosanoids at inflammatory sites as well as their kinetics should be taken into consideration in order to enhance our understanding of neutrophil involvement in shaping the inflammatory response. COX-2 is a multifaceted protein; in line with the proposed anti-apoptotic and growth-promoting properties of some of its products, the onset of COX-2 expression is likely to influence the functional activities of the neutrophil.

In summary, we have identified a number of inflammatory agents that, in vitro, rapidly induce the expression of COX-2 and the synthesis of TXA_2 and PGE_2 from endogenous AA in human neutrophils. The responses depend on signaling pathways, which rely on both transcriptional and translational events. Studies with selected pharmacological agents showed that regulation of the neutrophil COX-2 differs from that observed in other inflammatory cells, such as monocytes. The results presented here also suggest that the COX-2 isoform is preeminent over COX-1 for the stimulated production of COX products in human neutrophils. The timely onset of COX-2 in the neutrophil may significantly affect the way these cells influence the inflammatory response. FJ

This work was supported by grants from the Arthritis Society of Canada, from the Medical Research Council (MRC) of Canada, and from the NH and MRC of Australia. M.P. is the recipient of a fellowship from the MRC of Canada. The authors wish to thank Mr. Serge Picard and Ms. Line Bouchard for excellent technical assistance.

Note added in proof: Maloney et al. (65) recently reported, in agreement with our results, that inflammatory agonists can induce the expression of COX-2 in human neutrophils, and provided evidence which suggests that COX-2 expression is differently regulated in myeloid cells of different lineages.

REFERENCES

1. Cassatella, M. A. (1995) The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today* **16**, 21–26
2. Simmons, P. M., Salmon, J. A., and Moncada, S. (1983) The release of leukotriene B4 during experimental inflammation. *Biochem. Pharmacol.* **32**, 1353–1359
3. Pouliot, M., Mcdonald, P. P., Khamzina, L., Borgeat, P., and McColl, S. R. (1994) Granulocyte-macrophage colony-stimulating factor enhances 5-lipoxygenase levels in human polymorphonuclear leukocytes. *J. Immunol.* **152**, 851–858
4. Pouliot, M., Mcdonald, P. P., Borgeat, P., and McColl, S. R. (1994) Granulocyte macrophage colony-stimulating factor stimulates the expression of the 5-lipoxygenase-activating protein (FLAP) in human neutrophils. *J. Exp. Med.* **179**, 1225–1232
5. Zurier, R. B., and Sayadoff, D. M. (1975) Release of prostaglandins from human polymorphonuclear leukocytes. *Inflammation* **1**, 93
6. Goldstein, I. M., Malmsten, C. L., Kindahl, H., Kaplan, H. B., Radmark, O., Samuelsson, B., and Weissmann, G. (1978) Thromboxane generation by human peripheral blood polymorphonuclear leukocytes. *J. Exp. Med.* **148**, 787–792
7. Dvorak, A. M., Morgan, E., Schleimer, R. P., Ryeom, S. W., Lichtenstein, L. W., and Weller, P. F. (1992) Ultrastructural immunogold localization of prostaglandin endoperoxide synthase (cyclooxygenase) to non-membrane-bound cytoplasmic lipid bodies in human lung mast cells, alveolar macrophages, type II pneumocytes, and neutrophils. *J. Histochem. Cytochem.* **40**, 759–769
8. Herrmann, F., Lindemann, A., Gauss, J., and Mertelsmann, R. (1990) Cytokine-stimulation of prostaglandin synthesis from endogenous and exogenous arachidonic acids in polymorphonuclear leukocytes involving activation and new synthesis of cyclooxygenase. *Eur. J. Immunol.* **20**, 2513–2516
9. Patrignani, P., Panara, M. R., Greco, A., Fusco, O., Natoli, C., Iacobelli, S., Cipollone, F., Ganci, A., Créminon, C., Maclouf, J., and Patrono, C. (1994) Biochemical and pharmacological characterization of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases. *J. Pharmacol. Exp. Ther.* **271**, 1705–1712
10. Smith, W. L., Borgeat, P., and Fitzpatrick, F. A. (1991) The eicosanoids: cyclooxygenase, lipoxygenase and epoxygenase pathways. In *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E., and Vance, J., eds) pp. 297–325, Elsevier, New York.
11. Vane, J. R., and Botting, R. M. (1996) Mechanism of action of anti-inflammatory drugs. *Scand. J. Rheumatol.* **102**, 9–21
12. O'Banion, M. K., Sadowski, H. B., Winn, V., and Young, D. A. (1991) A serum- and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. *J. Biol. Chem.* **266**, 23261–23267
13. Smith, W. L., Garavito, R. M., and Dewitt, D. L. (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2 [Review]. *J. Biol. Chem.* **271**, 33157–33160

14. Crofford, L. J. (1997) Cox-1 and cox-2 tissue expression—implications and predictions. *J. Rheumatol.* **24**, 15–19
15. O'Banion, M. K., Winn, V. D., and Young, D. A. (1992) cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc. Natl. Acad. Sci. USA* **89**, 4888–4892
16. Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Lee, L., and Isakson, P. (1994) Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc. Natl. Acad. Sci. USA* **91**, 12013–12017
17. Dinchuk, J. E., Car, B. D., Focht, R. J., Johnston, J. J., Jaffee, B. D., Covington, M. B., Contel, N. R., Eng, V. M., Collins, R. J., Czerniak, P. M., Gorry, S. A., and Trzaskos, J. M. (1995) Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase 2. *Nature (London)* **378**, 406–409
18. Herschman, H. R. (1996) Prostaglandin synthase 2 [Review]. *Biochim. Biophys. Acta* **1299**, 125–140
19. Hla, T., and Neilson, K. (1992) Human cyclooxygenase-2 cDNA. *Proc. Natl. Acad. Sci. USA* **89**, 7384–7388
20. Masferrer, J. L., Zweifel, B. S., Manning, P. T., Hauser, S. D., Leahy, K. M., Smith, W. G., Isakson, P. C., and Seibert, K. (1994) Selective inhibition of inducible cyclooxygenase-2 in vivo is antiinflammatory and nonulcerogenic. *Proc. Natl. Acad. Sci. USA* **91**, 3228–3232
21. Tomlinson, A., Appleton, I., Moore, A. R., Gilroy, D. W., Willis, D., Mitchell, J. A., and Willoughby, D. A. (1994) Cyclo-oxygenase and nitric oxide synthase isoforms in rat carrageenin-induced pleurisy. *Br. J. Pharmacol.* **113**, 693–698
22. Niiro, H., Otsuka, T., Izuhara, K., Yamaoka, K., Ohshima, K., Tanabe, T., Hara, S., Nemoto, Y., Tanaka, Y., Nakashima, H., and Niho, Y. (1997) Regulation by interleukin-10 and interleukin-4 of cyclooxygenase-2 expression in human neutrophils. *Blood* **89**, 1621–1628
23. Boyum, A. (1968) Isolation of mononuclear cells and granulocytes from human peripheral blood. *Scand. J. Clin. Lab. Invest.* **21**, 77–89
24. Habib, A., Creminon, C., Frobert, Y., Grassi, J., Pradelles, P., and Maclouf, J. (1993) Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. *J. Biol. Chem.* **268**, 23448–23454
25. Margalit, A., Duffin, K. L., and Isakson, P. C. (1996) Rapid quantitation of a large scope of eicosanoids in two models of inflammation: development of an electrospray and tandem mass spectrometry method and application to biological studies. *Anal. Biochem.* **235**, 73–81
26. Pradelles, P., Grassi, J., and Maclouf, J. (1985) Enzyme immunoassays of eicosanoids using acetylcholine esterase as label: an alternative to radioimmunoassay. *Anal. Chem.* **57**, 1170–1173
27. Jones, D. A., Carlton, D. P., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1993) Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. *J. Biol. Chem.* **268**, 9049–9054
28. Otto, J. C., Dewitt, D. L., and Smith, W. L. (1993) N-glycosylation of prostaglandin endoperoxide synthases-1 and synthases-2 and their orientations in the endoplasmic reticulum. *J. Biol. Chem.* **268**, 18234–18242
29. Pouliot, M., Baillargeon, J., Lee, J. C., Cleland, L. G., and James, M. J. (1997) Inhibition of prostaglandin endoperoxide synthase-2 expression in stimulated human monocytes by inhibitors of p38 mitogen-activated protein kinase. *J. Immunol.* **158**, 4930–4937
30. Ridley, S. H., Sarsfield, S. J., Lee, J. C., Bigg, H. F., Cawston, T. E., Taylor, D. J., Dewitt, D. L., and Saklatvala, J. (1997) Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase—regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. *J. Immunol.* **158**, 3165–3173
31. Chabot, M. C., Mcphail, L. C., Wykle, R. L., Kennerly, D. A., and McCall, C. E. (1992) Comparison of diglyceride production from choline-containing phosphoglycerides in human neutrophils stimulated with N-formylmethionyl-leucylphenylalanine, ionophore A23187 or phorbol 12-myristate 13-acetate. *Biochem. J.* **286**, 693–699
32. Bonser, R. W., Thompson, N. T., Randall, R. W., and Garland, L. G. (1989) Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem. J.* **264**, 617–620
33. Glaser, K. B., and Lock, Y. W. (1995) Regulation of prostaglandin H synthase 2 expression in human monocytes by the marine natural products manoalide and scalaradial—novel effects independent of inhibition of lipid mediator production. *Biochem. Pharmacol.* **50**, 913–922
34. Angel, J., Berenbaum, F., Ledenmat, C., Nevalainen, T., Masliah, J., and Fournier, C. (1994) Interleukin-1-induced prostaglandin E2 biosynthesis in human synovial cells involves the activation of cytosolic phospholipase A2 and cyclooxygenase-2. *Eur. J. Biochem.* **226**, 125–131
35. Crofford, L. J., Wilder, R. L., Ristimaki, A. P., Sano, H., Remmers, E. F., Epps, H. R., and Hla, T. (1994) Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1 beta, phorbol ester, and corticosteroids. *J. Clin. Invest.* **93**, 1095–1101
36. Godessart, N., Camacho, M., Lopez-Belmonte, J., Anton, R., Garcia, M., Demoragas, J. M., and Vila, L. (1996) prostaglandin H-synthase-2 is the main enzyme involved in the biosynthesis of octadecanoids from linoleic acid in human dermal fibroblasts stimulated with interleukin-1-beta. *J. Invest. Dermatol.* **107**, 726–732
37. Dayton, E. T., and Major, E. O. (1996) Recombinant human interleukin 1-beta induces production of prostaglandins in primary human fetal astrocytes and immortalized human fetal astrocyte cultures. *J. Neuroimmunol.* **71**, 11–18
38. Albert, T. J., Su, H. C., Zimmerman, P. D., Iams, J. D., and Kniss, D. A. (1994) Interleukin-1-beta regulates the inducible cyclooxygenase in amnion-derived wish cells. *Prostaglandins* **48**, 401–416
39. Lyons-Giordano, B., Pratta, M. A., Galbraith, W., Davis, G. L., and Arner, E. (1993) Interleukin-1 differentially modulates chondrocyte expression of cyclooxygenase-2 and phospholipase A2. *Exp. Cell. Res.* **206**, 58–62
40. Vigano, T., Habib, A., Hernandez, A., Bonazzi, A., Boraschi, D., Lebret, M., Cassina, E., Maclouf, J., Sala, A., and Folco, G. (1997) Cyclooxygenase-2 and synthesis of PGE2 in human bronchial smooth-muscle cells. *Am. J. Resp. Critical Care Med.* **155**, 864–868
41. Akarasereenont, P., and Thiemermann, C. (1996) The induction of cyclo-oxygenase-2 in human pulmonary epithelial cell culture (A549) activated by IL-1-beta is inhibited by tyrosine kinase inhibitors. *Biochem. Biophys. Res. Commun.* **220**, 181–185
42. Rzymkiewicz, D., Leingang, K., Baird, N., and Morrison, A. R. (1994) Regulation of prostaglandin endoperoxide synthase gene expression in rat mesengial cells by interleukin-1β. *Am. J. Physiol.* **266**, F39–F45
43. Yucellindberg, T., Ahola, H., Nilsson, S., Carlstedtduke, J., and Modeer, T. (1995) Interleukin-1-beta induces expression of cyclooxygenase-2 mRNA in human gingival fibroblasts. *Inflammation* **19**, 549–560
44. Sato, T., Morita, K., Sakaguchi, K., Nakahama, K. I., Smith, W. L., Dewitt, D. L., and Murota, S. I. (1996) Involvement of prostaglandin endoperoxide H synthase-2 in osteoclast-like cell formation induced by interleukin-1-beta. *J. Bone Min. Res.* **11**, 392–400
45. Surette, M. E., Palmantier, R., Gosselin, J., and Borgeat, P. (1993) Lipopolysaccharides prime whole human blood and isolated neutrophils for the increased synthesis of 5-lipoxygenase products by enhancing arachidonic acid availability—involvement of the CD14 antigen. *J. Exp. Med.* **178**, 1347–1355
46. Karin, M., and Hunter, T. (1995) Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol.* **5**, 747–757
47. Masferrer, J. L., and Seibert, K. (1994) Regulation of prostaglandin synthesis by glucocorticoids. *Receptor* **4**, 25–30
48. Pilbeam, C. C., Kawaguchi, H., Hadeka, Y., Voznesensky, O., Alander, C. B., and Raisz, L. G. (1993) Differential regulation of inducible and constitutive prostaglandin endoperoxide synthase in osteoblastic MC3T3-E1 cells. *J. Biol. Chem.* **268**, 25643–25649
49. Potestio, F. A., Zakar, T., and Olson, D. M. (1988) Glucocorticoids stimulate prostaglandin synthesis in human amnion cells by a receptor-mediated mechanism. *J. Clin. Endocrinol. Metab.* **67**, 1205–1210
50. Zakar, T., and Olson, D. M. (1989) Dexamethasone stimulates arachidonic acid conversion to prostaglandin E2 in human cells. *J. Dev. Physiol.* **12**, 269–272

51. Zakar, T., Hirst, J. J., Mijovic, J. E., and Olson, D. M. (1995) Glucocorticoids stimulate the expression of prostaglandin endoperoxide H synthase-2 in amnion cells. *Endocrinology* **136**, 1610–1619
52. Ristimaki, A., Narko, K., and Hla, T. (1996) Down-regulation of cytokine-induced cyclo-oxygenase-2 transcript isoforms by dexamethasone—evidence for post-transcriptional regulation. *Biochem. J.* **318**, 325–331
53. Fletcher, B. S., Kujubu, D. A. , Perrin, D. M., and Herschman, H. R. (1992) Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin G/H synthase. *J. Biol. Chem.* **267**, 4338–4344
54. Sirois, J., Levy, L. O., Simmons, D. L., and Richards, J. S. (1993) Characterization and hormonal regulation of the promoter of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. Identification of functional and protein-binding regions. *J. Biol. Chem.* **268**, 12199–12206
55. Inoue, H., Yokohama, C., Hara, S., Tone, Y., and Tanabe, T. (1995) Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *J. Biol. Chem.* **270**, 24965–24971
56. Yamamoto, K., Arakawa, T., Ueda, N., and Yamatoto, S. (1995) Transcriptional roles of nuclear factor κB and nuclear factor-interleukin-6 in the tumor necrosis factor α-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J. Biol. Chem.* **270**, 31315–31320
57. Kosaka, T., Miyata, A., Ihara, H., Hara, S., Sugimoto, T., Takeda, O., Takahashi, E., and Tanabe, T. (1994) Characterization of the human gene (PTGS2) encoding prostaglandin-endoperoxide synthase 2. *Eur. J. Biochem.* **221**, 889–897
58. Xie, W., and Hershman, H. R. (1995) v-Src induces prostaglandin synthase 2 gene expression by activation of the c-Jun N-terminal kinase and the c-Jun transcription factor. *J. Biol. Chem.* **270**, 27622–27628
59. Della Bianca, V., Grzeskowiak, M., Lissandrini, D., and Rossi, F. (1991) Source and role of diacylglycerol formed during phagocytosis of opsonized yeast particles and associated respiratory burst in human neutrophils. *Biochem. Biophys. Res. Commun.* **177**, 948–955
60. Reinhold, S. L., Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1990) Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB J.* **4**, 208–214
61. Bauldry, S. A., Bass, D. A., Cousart, S. L., and McCall, C. E. (1991) Tumor necrosis factor alpha priming of phospholipase D in human neutrophils. Correlation between phosphatidic acid production and superoxide generation. *J. Biol. Chem.* **266**, 4173–4179
62. L'Heureux, G. P., Bourgoin, S., Jean, N., McColl, S. R., and Naccache, P. H. (1995) Diverging signal transduction pathways activated by interleukin-8 and related chemokines in human neutrophils: interleukin-8, but not NAP-2 or GRO alpha, stimulates phospholipase D activity. *Blood* **85**, 522–531
63. Sciorra, V. A., and Daniel, L. W. (1996) Phospholipase D-derived products in the regulation of 12-O-tetradecanoylphorbol-13-acetate-stimulated prostaglandin synthesis in Madin-Darby canine kidney cells. *J. Biol. Chem.* **271**, 14226–14232
64. Mollideno, F., Gajate, C., and Flores, I. (1994) Involvement of phospholipase D in the activation of transcription factor AP-1 in human T lymphoid Jurkat cells. *J. Immunol.* **153**, 2457–2469
65. Maloney, C. G., Kutchera, W. A., Albertine, K. H., Mcintyre, T. M., Prescott, S. M., and Zimmerman, G. A. (1998) Inflammatory agonists induce cyclooxygenase type 2 expression by human neutrophils. *J. Immunol.* **160**, 1402–1410

Received for publication February 4, 1998.
Revised for publication April 6, 1998.