Arachidonic Acid in Platelet Microparticles Up-regulates Cyclooxygenase-2-dependent Prostaglandin Formation via a Protein Kinase C/Mitogen-activated Protein Kinase-dependent Pathway*

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Activation of platelets results in shedding of membrane microparticles (MP) with potentially bioactive properties. Platelet MP modulate platelet, monocyte, and vascular endothelial cell function, both by direct effects of MP arachidonic acid (AA) and by its metabolism to bioactive prostanooids. We have previously reported that platelet MP induce expression of cyclooxygenase (COX)-2 and prostacyclin production in monocytes and endothelial cells. To elucidate further the molecular mechanisms that underlie MP-induced up-regulation of COX-2 expression, we investigated the response of a human monocytoid (U-937) cell line to platelet MP stimulation.

In U-937 cells, MP-induced COX-2 expression and eicosanoid formation is prevented by pharmacological inhibitors of protein kinase C (PKC), PI 3-kinase, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase, and p38 kinase. Treatment with the PI 3-kinase inhibitors wortmannin and LY294002 also blocked MP-induced p42/p44 MAPK, p38, and JNK1 phosphorylation. Conversely, platelet MP stimulation of U-937 cells results in direct activation of PKC, p42/p44 MAPK, p38 kinase, and c-Jun N-terminal kinase (JNK) as well as activation of the transcription factors c-Jun and Elk-1. However, MP failed to activate the cAMP response element.

Activation of U-937 cells by MP induces translocation of classical (PKCβ), novel (PKCδ) and atypical (PKCζ and PKCa) isoforms of PKC from the cytosol to the membrane, with concomitant activation of downstream MAPK. While MP-induced activation of p42/p44 MAPK and p38 kinase is transient, a sustained activation of JNK1 was observed. Although PKC activation is required for MP-induced p42/p44 MAPK, activation of the stress kinases p38 and JNK1 was PKC-independent. The fatty acid fraction of the MP accounted for these effects, which were mimicked by MP AA. Rather than acting directly via nuclear receptors, MP AA activates COX-2-dependent prostaglandin production by a PKC/p42/p44 MAPK/p38 kinase-sensitive pathway in which PI 3-kinase plays a significant role. MP AA also stimulates transcriptional activation of COX-2 as well as c-Jun and Elk-1.

Activation of platelets by agonists, such as collagen and thrombin, results in shedding of membrane microparticles (MP)† from their surface (1). Formation of MP results from an exocytotic budding process (2, 3) and may include both procoagulant (4, 5) and anticoagulant (6, 7) proteins when shed from platelets. Increased circulating concentrations of MP have been detected in vivo in syndromes of platelet activation (8, 9). It is likely that the shear forces in areas of disordered flow would favor MP formation (10, 11).

MP might themselves evoke cellular responses in the immediate microenvironment of their formation. For instance, activation of endothelial cells results in MP shedding, which, in turn, activates neutrophils, enhancing their propensity to adhere to endothelium (12). Recently, we have demonstrated that arachidonic acid (AA) in platelet MP can influence platelet activation in a PKC-dependent manner and endothelial cell COX-2 expression via transcellular lipid metabolism (13). The molecular mechanisms by which MP induce such cellular responses are, however, unknown. The present studies were designed to delineate the signaling pathways involved in MP-induced COX-2 activation and prostaglandin formation.

COX is the first rate-limiting enzyme in the synthesis of prostacyclin, prostaglandins, and thromboxane from arachidonic acid (14). Two isoforms of COX have been described (15, 16), and both catalyze the cyclooxygenase-dependent transformation of prostaglandin G2 from AA and the subsequent peroxidation of prostaglandin G2 to prostaglandin H2 (17). While COX-1 is expressed constitutively in most tissues, COX-2 is usually induced as an immediate early gene by mitogenic or inflammatory stimuli as well as by ligands that act via G protein- and PKC-mediated pathways (18). More recent findings link the prostaglandin biosynthetic pathways with activation of MAPK signaling cascades (19–21).

The MAPK pathway, of which there are three subgroups, is a conserved eukaryotic signaling cascade that is responsible for mediating the effects of extracellular stimuli on a wide variety of biological processes. The extracellular signal-regulated kinases (also termed p42 MAPK and p44 MAPK) and the stress-activated protein kinases (also termed c-Jun NH2-terminal kinase (JNK)) and p38 (22) are distinguished by activating signals, substrate specificity, and cellular responses (23). While

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the extracellular signal-regulated kinases are predominantly activated by growth factors (24, 25), the JNKs and p38 MAPKs are activated by stress signals such as inflammatory cytokines, heat shock, ultraviolet light, and ischemia (26).

It is thought that polyunsaturated fatty acids may influence gene regulation indirectly, via activation of membrane receptors coupled to downstream signaling cascades (27) or perhaps via nuclear receptors (28, 29). We have previously demonstrated that unmetabolized AA in platelet MP modulates adhensive interactions between monocytes and endothelial cells (30), a cellular interaction thought to be of relevance in the early stages of atherogenesis (31). Given that neither a membrane nor a nuclear receptor with high affinity for AA has been identified, we thought it of interest to delineate the molecular mechanisms that underlie this phenomenon.

In the present study, we show that MP AA mimics the effect of MP in inducing COX-2 expression and prostaglandin formation in U-937 cells. MP AA activates PKC, p42/p44 MAPK, and PI 3-kinase, and PI 3-kinase prevents MP-mediated COX-2 expression and prostaglandin formation. These observations suggest that platelet MP AA may activate a membrane-linked signaling cascade that culminates in expression of COX-2 rather than requiring activation of nuclear receptors to regulate gene expression.

MATERIALS AND METHODS

Reagents—Indomethacin, N,N-diisopropylthylamine, pentfluorophenyl benzyl bromide, EDTA, EGTA, cycloheximide, actinomycin-D, leupeptin, Nonidet P-40, soybean trypsin inhibitor, and aprotinin were purchased from Sigma. Phorbol 12-myristate-13-acetate (PMA), LYS294022, and wortmannin were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). The prostacyclin analog, iloprost, AA, [3H]AA, TxB2, [3H]TxB2, PGE2, and [3H]PGE2, were all purchased from Cayman Chemical Co. (Ann Arbor, MI). GP 109020X (bisindolylmaleimide I), N-[2-(p-bromocinnamylamino)-ethyl]-5-isouquinolinesulfonamide (H-89), SB 203580, and forskolin were purchased from Calbiochem. [3H]Orthophosphate (6000–7000 Ci/mmol) and [3H]ATP (3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Mouse monoclonal antibodies against COX-1 and COX-2 were kindly provided by the laboratories of J. F. Mallouf (INSERM, Paris, France). The COX-2 promoter construct was a kind gift from Dr. S. Prescott (University of Utah, Salt Lake City, UT). The affinity-purified antibodies against PKCβ, PKCδ, PKCγ, and PKCλ were purchased from Transduction laboratories (Lexington, KY). Phospholipase p42/p44 MAPK, and p38 MAPK antibody kits and the MEK-1 inhibitor 2-(2-sulfophenyl)-5-(N-morpholino)-oxanaphthalen-4-one (PD98059) were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antibody against nonphosphorylated JNK and mouse monoclonal antibody against phosphorylated JNK were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PathDetectTM in vivo signal transduction pathway trans-reporting system was purchased from Stratagene (La Jolla, CA). The luciferase assay kit and the pGEL2-Basic vector were purchased from Promega (Madison, WI).

Isolation of Platelets. Preparation of Platelet MP, and Their Identification—Platelets were obtained from healthy volunteers as described previously (13). The volunteers had not taken any medication for at least 14 days. Briefly, blood was collected into a plastic syringe containing 3.8% buffered sodium citrate as anticoagulant (ratio 1:9). Platelet-rich plasma was prepared by centrifugation at 130 × g for 15 min, and platelet-poor plasma by centrifugation of platelet-rich plasma at 900 × g. Washed platelets were isolated from platelet-rich plasma after centrifugation and resuspended in calcium- and magnesium-free Hepes. Washed platelets were isolated from platelet-rich plasma after centrifugation and resuspended in calcium- and magnesium-free Hepes. Washed platelets were isolated from platelet-rich plasma after centrifugation and resuspended in calcium- and magnesium-free Hepes.

Platelet aggregation was studied at 37 °C using washed platelets in a glass capillary tube described previously (13). Samples were analyzed on a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA), and each preparation was assayed to exclude endotoxin contamination, using the Limulus amebocyte lysate assay. Final endotoxin contamination was always <0.02 unit/mg of protein. MP were also preincubated with polymixin B (50 μg/ml) for 1 h before their addition to U-937 cells to exclude bacterial contamination.

Cell Culture—U-937 cells, obtained from the American Type Tissue Culture Collection (Rockville, MD), were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 μg/ml streptomycin, and 100 units of penicillin. They were kept in a humidified incubator with RPMI 1640, and 5 × 105 cells/well were plated into 12-well tissue culture plates. Cell viability was determined by the trypan blue exclusion test. Indomethacin (20 μM) was always present in the medium for all experiments described.

Isolation of Arachidonic Acid—AA isolation was performed as described previously (13). Briefly, lipids were extracted according to a modification of the method of Bligh and Dyer (32) and separated as described in our previous publication (13). The free fatty acid fraction was purified by amino (NH2) column chromatography (International Sorbert Technology, Glamorgan, United Kingdom) followed by TLC. The band with the same Rf as standard AA was extracted from the TLC plate and purified by reverse phase high performance liquid chromatography. Identification of AA was confirmed by negative ion chemical ionization mass spectrometry, as described previously (13).

Analysis of COX Expression—Whole blot analysis of COX proteins was carried out as described previously (13). Briefly, U-937 cells were stimulated with MP (6 μg/ml), the equivalent of 10 μM MP AA (3 μg/ml) and lysed following stimulation for 2, 6, 12, or 24 h. Equal amounts of protein (20 and 40 μg for COX-1 and COX-2, respectively) were separated by 12% SDS-PAGE and transferred to nitrocellulose. After blocking the membranes with 2% 3H skim milk in Tris-buffered saline (Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Tween 20), they were incubated with mouse monoclonal antibody directed against either COX-1 or COX-2 at a dilution of 1:10,000 for 1 h at room temperature. Both antibodies are specific for their respective enzymes, as described previously (33, 34). Membranes were incubated with sheep anti-mouse IgG conjugated with horseradish peroxidase at 1:2000 for 1 h at room temperature. Chemiluminescent substrates were used to reveal positive bands that were visualized after the exposure to Hyperfilm ECL (Amersham Pharmacia Biotech).

Gas Chromatography/Mass Spectrometry—GC/MS analysis studies were performed on a Fisons MD-800 GC/MS (VG Organic, Manchester, UK) equipped with a split/splitless injector operated in the splitless mode at 260 °C. The ions monitored were m/z 614 for TxB2, m/z 615 for [3H]TxB2, m/z 622 for [3H]TxB2, m/z 624 for PGE2, m/z 628 for [3H]PGE2, and m/z 303 for AA, and m/z 311 for [3H]TxA2 concentrations, which were analyzed by gas chromatography/mass spectrometry (GC/MS) as described below.

Evaluation of PKC Activity—Expression and purification of recombinant PKC isoforms are described elsewhere (35). The specific enzymatic activities of the PKC isoforms used in this study have been previously described (35). PKC activity was measured using a mixture of 50 mM Tris-HCl, pH 7.4, 2 mM EGTA, 50 mM ATP, [γ-32P]ATP (3 μCi/tube), 15 mM CH3OH, 10 μM α-casein substrate peptide (RFARGLSRLQKNV) as the phosphoacceptor, the corresponding PKC isoform, and increasing concentrations of AA. No PMA or phosphatidyserine (PS) was added. Assays were carried out at 30 °C for 5 min. Results are expressed and normalized as a percentage of the maximum stimulation observed for each PKC isoform. We selected 100 μg/ml PS alone as a PKC activator in the case of PKCδ and 100 μg/ml PS and 10 μM PMA for all other PKC isoforms (35).

Analysis of Translocation of PKC Isozymes—U-937 cells were treated with different concentrations of MP or MP AA. Cytosolic and particulate fractions were separated by ultracentrifugation as described by Szallasi et al. (36), and PKC was evaluated in both fractions by kinase activity or by Western blotting. Total PKC activity in each fraction was measured under continuous conditions of activation but with the addition of PS (100 μg/ml) and PMA (10 μM) to the mixture for maximal stimulation. For Western blot analysis, samples of the cytosolic and particulate fractions were subjected to immunoblot analysis using specific antibodies for each PKC isoform. Briefly, after incubation with MP or MP AA, U-937 cells were lysed in ice-cold buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 μg/ml 4-(2-aminophenoxy)-benzenesulfonyl fluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin. The cell lysate was centrifuged at 9,000 × g for 30 min, and the supernatant was collected to determine [3H]PGE2 and [3H]TxA2 concentrations, which were analyzed by gas chromatography/mass spectrometry (GC/MS) as described below.
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**Fig. 1.** MP and MP AA stimulate a time-dependent expression of COX-2, but not of COX-1, in a PKC-, MAPK-, p38 MAPK-, and PI 3-kinase-dependent manner in U-937 cells. U-937 cells were treated with MP (6 μg/ml) (A, B, and E) or MP AA (3 μg/ml) (C, D, and E) for 0, 2, 6, 12, or 24 h. U-937 cells were incubated with cycloheximide (CHX) (5 μg/ml) for 1 h before the addition of platelet MP or MP AA. Cells were co-incubated with 12 μm actinomycin D (ACT) and either platelet MP or MP AA. H-89 dihydrochloride (H89) (10 μM), GF 109203X (GF) (5 μM), PD98059 (PD) (10 μM), SB203580 (SB) (10 μM), LY294002 (LY) (20 μM), or wortmannin (Wort) (100 nM) were also added to the cells for 1 h before the addition of platelet MP or MP AA. Similar results were obtained in three additional experiments.
expression of COX-1 were observed after MP treatment (Fig. 1E). Expression of COX-2 protein coincided with an increase in PGE2 and TxB2 synthesis, as measured in the supernatant (Table I). This increase was dose-dependent (data not shown). The EC50 values were 3 and 6 μg/ml of MP protein for PGE2 and TxB2, respectively. Indomethacin (20 μM), which is a preferential inhibitor of COX-1, which is expressed constitutively in U-937 cells, was always present in the medium (39). Actinomycin D (12 μM) and cycloheximide (5 μg/ml), two inhibitors of protein synthesis, prevented MP-induced COX-2 expression (Fig. 1A) as well as production of PGE2 and TxB2 (Table III). L-745,337 (10 μM), a highly specific inhibitor of COX-2 activity (40), markedly reduced the ability of MP to augment PGE2 and TxB2 production (Table I).

MP AA Mimics the Effect of MP on COX-2 Expression—The fatty acid fraction isolated from MP was found to account for the increase in expression of COX-2. AA isolated from this fraction was found to mimic the kinetics of the COX-2 expression induced by intact MP (Fig. 1C). No changes in COX-1 expression were observed (Fig. 1E). To address further the likelihood that MP-dependent induction of COX-2 was mediated by AA itself and not by its prostaglandin metabolites, we co-incubated U-937 cells with a stable prostacyclin analogue, iloprost (100 nM), with U46619 (1 μM), and with PGE2 (10 μM). Although all three were found to induce COX-2 expression in U-937 cells, the kinetics of protein expression were substantially different from those evoked by MP. Maximum induction of COX-2 expression by both U46619 and PGE2 occurred at 6 h, while iloprost-induced COX-2 was maximal at 24 h (data not shown). The time course of COX-2 induction by AA, by contrast, corresponded to that evoked by platelet MP (Fig. 1C).

Since we have previously shown that MP donate AA for transcellular metabolism in endothelial cells (13), we decided to determine whether a similar transfer mechanism might occur in U-937 cells. When MP loaded with 10 μM AA was added to U-937 cells, both [3H]PGE2 and [3H]TxB2 were synthesized and released to the supernatant (Table II). Thus, MP AA may be used as a substrate for prostaglandin formation by MP-induced COX-2 in U-937 cells.

PKC, p42/p44 MAPK, p38 Kinase, and PI 3-Kinase Mediate MP-induced COX-2 Expression—To address the possible involvement of PKC in MP-induced U-937 COX-2 and prostaglandin formation, cells were pretreated with GF 109203X (5 μM), a PKC inhibitor (41). GF 109203X abolished the MP and the MP AA effect on COX-2 expression, while the PKA inhibitor H-89 (10 μM) was without effect (Fig. 1, A and C). However, since GF 109203X does not inhibit either PKCe or PKCa, they are unlikely to be involved in MP-induced COX-2 expression. GF 109203X also inhibited the synthesis of PGE2 and TxB2 induced by MP, while H-89 was again ineffective (Table III). Cells were incubated with PD98059 (10 μM) and SB203580 (10 μM), inhibitors of MEK1 (42) and p38 kinase (43), respectively, as a first approach to investigate whether the MAPK cascade could play a potential role in MP- and MP AA-induced COX-2 expression and prostaglandin formation. Pretreatment with each of these inhibitors resulted in down-regulation of MP and MP AA-induced COX-2 expression (Fig. 1, B and D) and prostaglandin formation (Table III). Likewise, pretreatment of U-937 cells with the PI 3-kinase inhibitors LY294002 (20 μM) and wortmannin (100 nM) (44) abolished the expression of COX-2 induced by either MP (Fig. 1B) and MP AA (Fig. 1D) as well as prostaglandin formation (Table III).

MP Induce PKC Isozyme Activation—The observation that GF 109203X prevented MP- and MP AA-induced COX-2 and prostaglandin formation suggested that MP treatment may lead to PKC activation. We examined the effect of MP on the translocation of PKC isozymes from cytosolic to particulate fractions to address further this hypothesis (45–47). Platelet MP markedly altered the distribution of PKC, as judged by the evaluation of kinase activity in subcellular fractions. Most of the PKC activity (approximately 70%) was present in the cytosolic fraction. Total PKC activity in the cytosolic fraction was reduced by 72% (at the highest concentration of MP tested (100 μg/ml)) (Fig. 2A).

Since PKC comprises a family of at least 11 isozymes with differences in regulation and subcellular distribution, we evaluated the translocation of individual PKC isozymes by Western blot analysis. While previous studies have identified PKCe, PKCε, and PKCζ isozymes in U-937 cells (48), we found that they also express PKCδ and PKCa. Very low levels of PKCγ were also detected (data not shown). PKCβ (a classical or calcium-dependent PKC), PKCδ (a novel or calcium-independent PKC), and PKCζ and PKCα (atypical or phorbol ester/diacylglycerol-insensitive PKCs), translocate from the soluble to the particulate fraction upon MP stimulation in a conccen-
tation-dependent fashion (Fig. 3A). No changes were observed in the content of total PKC isozymes in total lysates. Densitometric analysis of the bands revealed a loss of 94 ± 4%, 75 ± 3%, 70 ± 6%, and 92 ± 2% (n = 4) for cytosolic PKCβ, PKCδ, PKCλ, and PKCζ, respectively, at the highest concentration (100 μg/ml) of MP tested (Fig. 3B). Therefore, translocation of U-937 cell PKC isozymes by platelet MP does not appear to discriminate among the different PKC subtypes present in the cell.

We studied the phosphorylation of PKC substrates to investigate further MP-induced PKC activation. Phosphorylation in intact cells has also been employed as an index of PKC activation. It was previously reported that treatment of monocytes and U-937 cells with PKC activators increases the phosphorylation of substrates of 120, 85, and 28 kDa (49, 50). There was a marked increase in the phosphorylation state of each of the three substrates by 312, 15, 441 ± 21, and 185 ± 30% (n = 4), respectively, in U-937 cells radiolabeled with [32P]orthophosphate, 10 min after stimulation with MP (30 μg/ml) (data not shown). Similar phosphorylation was observed when the cells were treated with PMA (100 nM) for 10 min. The phosphorylation observed after MP or PMA treatment was prevented by pretreatment with the PKC inhibitor GF 109203X (5 μM) but not by the PKA inhibitor H-89 (10 μM) (data not shown).

**MP AA Mimics the Effect of MP on PKC Activation**—Micromolar concentrations of AA and other unsaturated fatty acids can directly activate PKC, at least in vitro (51). We have previously shown that MP AA mediates PKC-dependent platelet aggregation (13). We treated U-937 cells with AA purified from MP with AA, fractionated the cells by ultracentrifugation, and measured total PKC activity in the soluble (cytosolic) and particulate fractions, as observed from the cytosolic to the particulate fractions (Fig. 4A). Densitometric analysis of the bands revealed a loss of immunoreactivity from the cytosolic fractions of 75 ± 9, 57 ± 2, 50 ± 3, and 82 ± 8% for PKCβ, PKCδ, PKCλ, and PKCζ, respectively (Fig. 4B). No changes in PKC activity were observed in aliquots of total lysates.

The effect of MP AA on PKC activation observed in U-937 cells was corroborated by  

**TABLE III**

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<th>MP</th>
<th>CHX</th>
<th>ACT</th>
<th>GF</th>
<th>H89</th>
<th>PD</th>
<th>SB</th>
<th>LY</th>
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<tr>
<td>PGE₂</td>
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<td>49 ± 7*</td>
<td>31 ± 5*</td>
<td>49 ± 13*</td>
<td>943 ± 83</td>
<td>120 ± 43*</td>
<td>168 ± 61*</td>
<td>78 ± 34*</td>
<td>66 ± 12*</td>
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<tr>
<td>TxB₂</td>
<td>5580 ± 930</td>
<td>60 ± 10*</td>
<td>50 ± 10*</td>
<td>30 ± 10*</td>
<td>5330 ± 1020</td>
<td>68 ± 23*</td>
<td>112 ± 54*</td>
<td>233 ± 101*</td>
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**Fig. 2.** MP and MP AA induce translocation of PKC activity. U-937 cells (5 × 10⁶ cells/ml) were stimulated with MP (1–100 μg/ml) (A) or MP AA (100 μM) (B) for 10 min. Kinase assays were carried out at 30 °C for 5 min. Cells were fractionated by ultracentrifugation for 1 h at 100,000 × g, and samples were assayed for PKC activity as described under “Materials and Methods.” Results are expressed as mean ± S.E. from three independent experiments.
AA was capable of stimulating PKCβ, -δ, and -ζ in a concentration-dependent fashion (Table IV). We did not study MP AA activation of PKCλ, since the purified enzyme was not available. A similar pattern of activation was observed with other PKC enzymes, such as PKCα, which are not present in U-937 cells (data not shown).

In vitro assays, using U-937 cells as a source of PKC, also revealed that MP AA induces activation of total PKC in U-937 cells (Table IV). Similar results in U-937 cells and in in vitro kinase assays were observed with AA obtained from a commercial source (Sigma) (data not shown). The PKC inhibitor, GF 109203X (5 μM), totally abolished MP-induced activation of U-937 PKC and recombinant PKCβ and PKCδ. PKCζ is insensitive to PKC inhibitors known to interfere with the catalytic domain of this enzyme (Ref. 41, and data not shown).

**MP Induce Activation of p42 and p44 MAPK**—Since PD98059, a specific inhibitor of MEK (42), prevented MP and MP AA-induced COX-2 activation (Fig. 1, B and D), we turned our attention to the MAPK signaling cascade. We studied the phosphorylation of MAPK in cell lysates to determine whether MP-induced activation of COX-2 was consistent with upstream activation of MAPK. We studied the phosphorylation of MAPK using phosphospecific antibodies as an index of MAPK activation (52–54). Phosphorylation of both p42 and p44 MAPK by MP (30 μg/ml) was evident as early as 5 min; this declined by 30 min and returned to basal levels at 1 h (Fig. 5B). Total
MAPK levels were not changed, as judged with a MAPK antibody that recognized both the phosphorylated and unphosphorylated forms of the enzyme (Fig. 5A).

We addressed the hypothesis that PKC influenced MP-induced phosphorylation of both p42 and p44 MAPK by pretreating the cells with GF 109203X (5 μM) for 1 h prior to stimulation. GF 109203X completely inhibited MP-induced phosphorylation of p42 and p44 MAPK (Fig. 5B). Likewise, staurosporine (100 nM), a nonspecific PKC inhibitor, also inhibited MP-induced p42/p44 MAPK phosphorylation (data not shown). Again, it seems likely that PKCl and PKCζ are not involved, since neither is inhibited by GF 109203X. Pretreatment of the cells with PMA for 48 h down-regulated PKC (55) and also totally inhibited the activation of p42/p44 MAPK by
MP (Fig. 5B). Pretreatment of the cells for 1 h with PD98059 (10 μM), an inhibitor of MEK1 (42), was also found to abolish completely MP-induced p42/p44 MAPK activation (Fig. 5B). To investigate whether PI 3-kinase is upstream or downstream of PKC in our system, we pretreated cells with LY294002 before stimulation with PMA. LY294002 failed to inhibit MP-induced p42/p44 MAPK phosphorylation, indicating that PI 3-kinase resides upstream of PKC (Fig. 6D).

**MP Induce Activation of p38 Kinase and JNK—**SB203580, a specific inhibitor for p38 activation (43), suppressed MP- and MP AA-induced COX-2 activation (Fig. 1, B and D). This result suggests that more than one MAPK cascade could be activated by MP treatment. Therefore, we evaluated the activation of p38 and JNK, using a similar approach as that used for p42/p44 MAPK. MP induced the phosphorylation of p38 (Fig. 6B). Activation of p38 was transient, similar to MP-induced p42/p44 MAPK activation. Maximal phosphorylation was observed at 5 min, which returned to near basal levels by 1 h post-MP treatment. Unlike MP-induced p42/p44 phosphorylation, however, MP-induced p38 phosphorylation is PKC-independent, since GF 109203X (5 μM) was without effect (Fig. 6B). Likewise, staurosporine (100 nM), was ineffective in preventing MP-induced p38 MAPK activation (data not shown). Pretreatment of the cells with SB203580 (10 μM) had no effect on MP-induced phosphorylation of p38 (Fig. 6B) as described previously by Cuenda et al. (43). Similarly, the MEK1 inhibitor PD98059 (10 μM), failed, as expected, to inhibit MP-induced p38 phosphorylation (data not shown). Total p38 protein levels were not changed by the MP treatment (Fig. 6A).

MP also induced the phosphorylation of JNK. The dominant form of JNK in U-937 cells is the 46-kDa form or JNK1 (Fig. 7A) as previously reported (56). When U-937 cells were treated with MP (30 μg/ml), the level of phosphorylated JNK1 was significantly elevated within 5 min, and remained so for 9 h (Fig. 7B). Pretreatment of the cells with GF 109203X (5 μM) did not inhibit MP-induced activation of JNK1 (Fig. 7B). A similar effect was observed with staurosporine (100 nM) (data not shown). No pharmacological inhibitors of the JNK pathway were available to our knowledge.

Inhibition of PI 3-kinase activity prevented MP and MP AA-induced COX-2 activation (Fig. 1, B and D). To determine whether PI 3-kinase activation is relevant to MP-induced p42/p44 MAPK, p38, and JNK activation, we used the same two pharmacological inhibitors, wortmannin and LY294002 (44). Both inhibitors reduced MP-induced activation of p42/p44 MAPK, p38, and JNK activation (Figs. 5B, 6B, and 7B), suggesting that PI 3-kinase is upstream of the MAPK cascades when U-937 cells are activated by MP.

**MP AA Mimics the Effect of MP on MAPK Activation—**U-937 cells were stimulated with MP AA (30 μM) for the same time periods as for intact MP. The same pattern of activation was observed for each of the kinases assayed. Maximal activation of p42/p44 MAPK and p38 by MP AA was observed at 5 min, declined at 30 min, and reached nearly basal levels by 1 h (Figs. 5C and 6C, respectively). Similarly, MP AA also mimicked JNK activation by intact MP (Fig. 7C). Pharamacological inhibition of PKC, MEK1, p38 MAPK, and JNK cascade had similar effects with MP AA as observed for intact MP, albeit the effects of inhibition of PI 3-kinase on p38 MAPK were somewhat less pronounced in the case of MP AA (Figs. 5C, 6C, and 7C).

**MP and MP AA Induce Transcriptional Activation of COX-2,** c-Jun, and Elk-1 but Not CRE—We investigated whether MP might influence gene regulation to evaluate further their effects on transcriptional activation of the COX-2 gene. When transfected U-937 cells were stimulated with MP (30 μg/ml), COX-2 activity increased significantly (Fig. 8). This was evident as early as 30 min and peaked at 4 h. A similar pattern of activation was observed when cells were stimulated with MP AA (30 μM) (Fig. 8). U-937 cells transfected with the pGL2-Basic vector, which lacks the promoter region of COX-2, showed little or no luciferase activity (Fig. 8). Furthermore, we also investigated whether MP could induce c-Jun or Elk-1 transcriptional activation. The COX-2 promoter has a CRE to which c-Jun binds (57). Our observations suggested that Elk-1, a substrate for both extracellular signal-regulated kinases and JNKs, might also regulate COX-2 expression (58, 59). Both MP and MP AA induction of c-Jun and Elk-1 was maximal at 4 h, similar to the kinetics of transcriptional activation of COX-2 by MP or MP AA (Fig. 8). U-937 cells cotransfected with the pFRLuc reporter plasmid and the pFC-Idb plasmid showed considerable increases in the luciferase activity.
little or no luciferase activity (Fig. 8). Both MP and MP AA induced the transcriptional activation of both c-Jun and Elk-1 as expected. A cross-talk mechanism has been previously reported between the MAPK and JNK signal transduction pathways in U-937 cells (60). We analyzed MP- and MP AA-induced activation of CRE (which is specifically activated by cAMP) by two methods to test the specificity of our system. In both cases, we failed to see an increase in luciferase activity, while forskolin, which increases cAMP levels, induced maximal CRE activation at 14 h in both systems (Fig. 8). This is consistent with our earlier results with the PKA inhibitor H-89, which failed to inhibit both MP- and MP AA-induced COX-2 expression (Fig. 1). Thus, MP- and MP AA-induced transcriptional activation is selective, but the increase in c-Jun activation may not be related to COX-2 induction in our system.

Activation of p42/p44 MAPK, p38, JNK phosphorylation, and CRE as well as transcriptional activation of c-Jun, Elk-1, or COX-2 by MP AA was not altered by indomethacin (20 μM), a nonspecific inhibitor of COX isoforms. Thus, AA, rather than its COX metabolites, predominantly mediates the effects of the lipid fraction of platelet MP on COX-2 transcription.

**DISCUSSION**

MP shed from activated platelets have been shown to influence cellular function. They circulate in syndromes of platelet activation (61) and may accumulate in areas of disordered blood flow (11). Recently, we have demonstrated that platelet-derived MP may facilitate transcellular delivery of AA, thereby modulating aspects of vascular and platelet function (13). The mechanisms by which MP activate cells remain poorly defined. Platelet MP influence adhesive interactions between monocytes and endothelial cells (30), a mechanism of potential relevance to initiation of vascular injury at sites of disordered laminar blood flow in the early stages of atherogenesis (31).

We have previously shown that MP induce human umbilical vein endothelial cell expression of COX-2 (13). Similarly, we now demonstrate that platelet MP and MP AA induce COX-2 and prostaglandin formation in U-937 monocytoid cells. The signaling pathways that regulate COX-2 expression are incompletely understood but may differ for the same agonist between cells and between agonists with a single cell type (62–64). It is unknown how AA regulates COX-2 expression. Indeed, since membrane receptors for AA remain to be identified, it is possible that it may ligate directly nuclear receptors that would...
regulate gene transcription. We now demonstrate that MP AA activates PKC isozymes as well as all three subgroups of the MAPK cascade. These events culminate in transcription factors of relevance to expression of COX-2.

MP and MP AA induce PKC isozyme phosphorylation and translocation as well as direct activation in vitro. Nishizuka and co-workers have previously demonstrated that micromolar concentrations of AA can activate classical, calcium-dependent PKCs (65). Given that both MP AA and MP induce an elevation in intracellular calcium in U-937 cells (data not shown) and that calcium has previously been shown to enhance AA-induced activation of PKC (65), it may be that classical PKCs are the predominant contributors to PKC-dependent effects of platelet MP. However, our findings clearly demonstrate that MP AA also activate novel and atypical PKC isoforms in U-937 cells.

In more recent years, a role for AA in the regulation of MAPK activation as well as transcriptional activation of c-Jun and Elk-1 has been proposed (66–68). The kinetics of MP AA-induced activation of PKC isozymes as well as all three subgroups of the MAPK pathway. For example, Xie et al. provided evidence for the role of JNK and c-Jun in mediating v-Src-induced COX-2 gene expression (19). Similarly, p38 kinase has been implicated in interleukin-1-stimulated COX-2-dependent prostaglandin production in fibroblasts, human umbilical vein endothelial cells, and mesangial cells (20, 21). Other elements of the prostaglandin pathway have also been linked with activation of MAPKs. For example, both MAPK and p38 mediate biosynthetic activation of cytosolic phospholipase A2 (71, 72).

We have demonstrated that MP AA can activate three distinct MAPKs (p42/p44 MAPK, p38, and JNK) in monocytic cells. Inhibitors of PI 3-kinase inhibit activation of all three kinases by MP AA, whereas inhibitors of PKC inhibit activation of p42/p44 MAPK but not p38 or JNK. The mechanisms involved in MP and MP AA activation of PI 3-kinase are under current investigation. Given that PKC inhibition markedly reduces MP-induced COX-2-dependent prostaglandin formation, it would seem that the p38 and JNK cascades play a less important role in this consequence of U-937 cell activation by platelet MP. We also demonstrate that both MP and MP AA can dramatically induce the transcriptional activation of the COX-2 gene. However, since MP and MP AA both failed to induce CRE activation, c-Jun, despite its activation by both MP and MP AA, is unlikely to influence COX-2 transcription in our system. The role of Elk-1 remains to be determined. Although we have focused on MP AA, we do not exclude the possibility of a role for other constituents of the fatty acid fraction in MP that may be of relevance to the regulation of COX-2. A preliminary model of events stimulated by platelet MP is provided in Fig. 9.

The present observations may also have relevance to the role of platelet activation in atherogenesis. Thus, laminar shear has been shown to produce prolonged up-regulation of COX-2 in endothelial cells (73). Production of prostacyclin, the predominant COX-2 product in these cells, inhibits platelet activation and adhesive interactions between circulating blood cells and the endothelium (74). Turbulent shear, by contrast, fails to up-regulate COX-2 in endothelial cells. A similar discrepancy of effect is observed with induction of endothelial nitric-oxide synthase (73). Given that turbulence may mimic the disordered laminar shear that occurs in vivo at sites of lesion development,
this raises the possibility that a localized deficiency in prostacyclin and NO may be of functional relevance to the focal nature of atherogenesis. Thus, disordered shear force favor platelet activation and the shedding of MP, an effect that can only be amplified by a localized, coexistent deficiency of endogenous platelet inhibitors, such as prostacyclin and NO. We have shown that MP AA can stimulate directly platelet aggregation and adhesive interactions involving both platelets and monocytes with endothelial cells. Furthermore, while disordered shear may impair the ability of endothelial cells to generate COX-2-dependent prostacyclin, stimulation of monocyte and platelet TxA2 by platelet MP would amplify further platelet activation and the adhesive interactions induced by MP AA.

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Arachidonic Acid in Platelet Microparticles Up-regulates Cyclooxygenase-2-dependent Prostaglandin Formation via a Protein Kinase C/Mitogen-activated Protein Kinase-dependent Pathway
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