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Matrix Metalloproteinase-Dependent Microsomal Prostaglandin E Synthase-1 Expression in Macrophages: Role of TNF-α and the EP4 Prostanoid Receptor

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Matrix metalloproteinase (MMP)-9 contributes to the pathogenesis of chronic inflammatory diseases and cancer. Thus, identifying targetable components of signaling pathways that regulate MMP-9 expression may have broad therapeutic implications. Our previous studies revealed a nexus between metalloproteinases and prostanoids whereby MMP-1 and MMP-3, commonly found in inflammatory and neoplastic foci, stimulate macrophage MMP-9 expression via the release of TNF-α and subsequent induction of cyclooxygenase-2 and PGE_2 engagement of EP4 receptor. In the current study, we determined whether MMP-induced cyclooxygenase-2 expression was coupled to the expression of prostaglandin E synthase family members. We found that MMP-1- and MMP-3-dependent release of TNF-α induced rapid and transient expression of early growth response protein 1 in macrophages followed by sustained elevation in microsomal prostaglandin synthase 1 (mPGES-1) expression. Metalloproteinase-induced PGE_2 levels and MMP-9 expression were markedly attenuated in macrophages in which mPGES-1 was silenced, thereby identifying mPGES-1 as a therapeutic target in the regulation of MMP-9 expression. Finally, the induction of mPGES-1 was regulated, in part, through a positive feedback loop dependent on PGE_2 binding to EP4. Thus, in addition to inhibiting macrophage MMP-9 expression, EP4 antagonists emerge as potential therapy to reduce mPGES-1 expression and PGE_2 levels in inflammatory and neoplastic settings.

pression, EP4 antagonists emerge as potential therapeutic agents to reduce mPGES-1 expression and PGE₂ levels in inflammatory or neoplastic settings.

**Materials and Methods**

**Macrophages**

Thiglycolate-elicited peritoneal macrophages were obtained from Swiss Webster mice by the method of Edelson and Cohn (26) as described previously (27). Mice were injected i.p. (3 ml/mouse) with 3% Brewer Thiglycolate Medium (Difco). Four days later, cells were harvested by lavage with cold Dulbecco’s PBS. Peritoneal cells were recovered by centrifugation and resuspended in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 mM glutamine and plated into multiwell plates. Cells were allowed to adhere for 4 h and then washed free of nonadherent cells. For serum-free conditions, macrophages were grown in DMEM supplemented with antibiotics, glutamine, and 0.1% BSA (Sigma-Aldrich) containing low levels of endotoxin (≤0.1 ng/ml; low endotoxin-BSA [LE-BSA]). Cellgro DMEM and heat-inactivated FBS were obtained from Mediatech Antibiotics, and glutamine was obtained from either Mediatech or Life Technologies. The murine macrophage cell line RAW264.7 (28) was obtained from American Type Culture Collection and maintained as adherent cultures in DMEM–10% FBS. All antibiotics, and glutamine were obtained from Mediatech. Antibiotics, and glutamine were obtained from Mediatech. The resulting PGE2 levels in macrophage conditioned media were determined and normalized for levels of actin mRNA, which served as a loading control. Data are reported as RDUs.

**Preparation of cell lysates**

Cell lysates were used in the analysis of Cox-2 expression were prepared by adding 1× SDS sample buffer directly to washed macrophage monolayers. In experiments designed to monitor levels of phosphorylated and total MAPK(erk)1/2, cells were lysed in TBS containing 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 1 mM PMSF, and 10 μg/ml aprotinin. Lysates were centrifuged (14,000 × g) for 20 min at 4°C. The supernatants were recovered, normalized for protein, and mixed with SDS sample buffer with 2-mercaptoethanol and boiled for 5 min. Equal amounts of cell lysates were applied to gels based on protein content.

**Western blots**

Cell lysates were electrophoresed in 4–15% polyacrylamide gels, and proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% defatted milk in TBST, washed in TBST, and incubated 18 h in blocking buffer containing affinity purified goat IgG raised against a peptide from the C terminus of human Cox-2 (0.2 μg/ml; Santa Cruz Biotechnology) or rabbit anti-phosphospecific p44/p42 MAPK (i.e., MAPK(erk)1/2) IgG (75 ng/ml; Cell Signaling Technology). Membranes were washed twice in TBST and incubated 1 h in blocking buffer containing affinity purified rabbit anti-goat IgG conjugated to HRP (0.1 μg/ml; Santa Cruz Biotechnology) or rabbit anti-goat IgG conjugated to HRP (0.3 μg/ml; Bio-Rad Laboratories). The membranes were washed three times in TBST, and bound HRP was visualized utilizing chemiluminescence (Pierce/Thermo Scientific). In the case of membranes probed with anti-phosphospecific MAPK(erk)1/2, after visualization of bound HRP, the membranes were stripped in 62.5 mM Tris buffer (pH 6.7) containing 100 mM Na2-mercaptoethanol and 2% SDS for 30 min at 50°C, washed, and probed for total MAPK(erk)1/2 (Cell Signaling Technology).

Determination of mPGES-1 mRNA in macrophage conditioned media

The concentrations of mPGES-1 in conditioned media were determined using the PGE2 enzyme immunoassay kit (monoclonal; Cayman Chemical).

**Evaluation of cellular toxicity**

To rule out toxic effects of the inhibitors used in these studies, cellular morphology and/or protein content after incubation are regularly monitored. In addition, we test for potential cytotoxicity by monitoring mitochondrial dehydrogenase activity using an MTT assay kit (Sigma-Aldrich). Levels of

**Gene knockdowns**

mPGES-1. RAW264.7 macrophages were transfected with ON-TARGETplus SMARTpool mPGES-1 siRNA or nonspecific siRNA (Dharmacon/Thermo Scientific) using HiPerFect transfection reagent according to the manufacturer’s recommended protocols (Qiagen). Cells were plated into a 12-well plate (2.5 × 10⁵ cells/well) and incubated 24 h with transfection complexes before exposure to MPPs or LPS.

TNF-α. RAW264.7 macrophages were transfected with TNF-α siRNA or nonspecific siRNA (Santa Cruz) according to the manufacturer’s protocol, as described previously (17).

EP4. Macrophages were transfected with siGENOME SMARTpool EP4 or nonspecific siRNA (Thermo Scientific/Dharmacon) using Gene Porter 3000 transfection reagent (GenLantis/Gen Therapy Systems) according to the manufacturer’s instructions. Macrophages were aliquoted into a 12-well plate (2.5 × 10⁶ cells/well) in antibiotic-free DMEM–10% FBS and incubated 18 h. The next day, the transfection lipoexolpes containing 50 mL siRNA were added to the cells and incubated 4 h. After the initial 4-h incubation, an equal volume of DMEM–20% FBS was added, and cells were incubated an additional 48 h. For each gene, PCR was used to determine the extent of knockdown.

**PCR analysis**

RNA was prepared using TRizol regent kits (Invitrogen). RNA (2 μg) was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo d(T)16 primer or random hexamers. The resulting cDNA was then used for amplification. The primers and conditions for PCR analysis of murine Cox-2, MMP-9, EP4, and actin have been previously described (29). Primers for murine mPGES-1, mPGES-2, and Egfr were designed using the Primer 3 program and were ordered from Sigma. The resulting PCR products were verified by sequencing (Cornell University Sequencing Facility). The primers for mPGES-1 were as follows: forward 5'-CCTTTGACTGCACTGCCTAC-3' (nucleotides 1484–1503) and reverse 5'-CAGCTAATGTTCAGCCAAGC-3' (nucleotides 2048–2069). Primers for mPGES-2 were as follows: forward 5'-ACTTTACCTTCGCTCGCTATA-3' (nucleotides 744–763) and reverse 5'-GCCCTACCGACAATGTAATG-3' (nucleotides 1186–1167). The primers for EP4 were as follows: forward 5'-CTCCCTCAGTCGTGTAACG-3' (nucleotides 552–573) and reverse 5'-CAGGTCTACCTTCCAGAGT-3' (nucleotides 826–805).

DNA (1–2 μM) were added to Platinum PCR SuperMix (final volume 25 μl; Invitrogen) in a thermocycler under the following conditions. mPGES-1: denature for 30 s at 94°C, anneal for 30 s at 55°C, and extend for 1 min at 72°C; mPGES-2: denature for 20 s at 94°C, anneal for 20 s at 65°C, and extend for 30 s at 72°C; repeat for 35 cycles with a final extension for 10 min at 72°C. Egr-1: denature for 20 s at 94°C, anneal for 20 s at 65°C, and extend for 30 s at 72°C; repeat for 35 cycles with a final extension for 10 min at 72°C. PCR products were electrophoresed in a 1% agarose gel with either 0.5 μg/ml ethidium bromide or 1× GelRed (Biotium; Hayward, CA) and photographed under UV light. PCR conditions for the target genes were optimized to ensure that experimental samples, excluding exuberant positive controls, are in the linear range when scanned. Densitometric analysis of the scanned target mRNAs were determined and normalized for levels of actin mRNA, which served as a loading control. Data are reported as RDUs.

For real-time PCR analysis, the reaction volume was 20 μl and contained 5 μl cDNA, 2× SYBR Green PCR master mix, and forward and reverse primers as described previously (30). Real-time PCR primers for mPGES-1 were as follows: forward 5'-TCCATTGGGACCACTTACAC-3' (nucleotides 730–749) and reverse 5'-ATGGCTCTGTGTTGCACTCC-3' (nucleotides 844–863). Primers for GAPDH were as follows: forward 5'-AATGGT-TCCGGTCTGAGTAC-3' (nucleotides 759–778) and reverse 5'-CATCGAACGAGAGAGAGG-3' (nucleotides 914–933). Levels of mPGES-1 mRNA were normalized to GAPDH mRNA.

**Evaluation of cellular toxicity**

To rule out toxic effects of the inhibitors used in these studies, cellular morphology and/or protein content after incubation are regularly monitored. In addition, we test for potential cytotoxicity by monitoring mitochondrial dehydrogenase activity using an MTT assay kit (Sigma-Aldrich). Levels of...
mitochondrial dehydrogenase activity were not significantly reduced after exposure to any of the inhibitors over the time periods described in the article.

Statistics

Mean RDUs for various genes and proteins and levels of PGE2 in cellular conditioned media were compared using single- or two-factor ANOVA, depending on the particular experimental design. After the determination of significant differences between the means of a given experiment, subsequent pairwise comparisons were performed using Newman–Keuls multiple range testing (31).

Results

**MMP-1 and MMP-3 induce mPGES-1 in macrophages**

Cox-2–derived PGH2 is converted to PGE2 by a family of PGE synthases including mPGES-1, mPGES-2, and cPGES (19). In studies reported here, we determined whether MMP-induced Cox-2 expression in macrophages and elevated PGE2 secretion (17) were coupled to changes in expression of these PGE synthases. Incubation with LPS, a potent inducer of mPGES-1 in macrophages, served as a positive control (22, 23, 32). Levels of mPGES-1 and mPGES-2 mRNA were monitored using semi-quantitative PCR, and levels of cPGES were determined using Western blot. As seen in Fig. 1A, treatment of RAW264.7 macrophages with active MMP-1 and MMP-3 induced mPGES-1 ∼3- and 6-fold (p < 0.005), respectively. In contrast, levels of mPGES-2 mRNA or cPGES Ag, which were constitutively expressed in control cells, were unchanged.

To corroborate these findings, we examined the effect of active MMPs on the expression of PGE synthases by macrophages recovered from thioglycolate-induced peritonitis (Fig. 1B). mPGES-1 mRNA levels were barely detected in the elicited macrophages. Incubation with MMP-1 and MMP-3 strongly induced macrophage expression of mPGES-1 (p < 0.02), whereas levels of mPGES-2 mRNA and cPGES Ag were unchanged. These data are consistent with reports that mPGES-1 is inducible and plays a major role in the elevation of PGE2 production observed in sites of inflammation (22–25, 33).

**Macrophage MMP-9 expression is dependent on inducible mPGES-1 expression**

To determine whether mPGES-1 played a principal role in proteinase-induced MMP-9 expression, mPGES-1 expression was knocked down using siRNA. For this purpose, we initially evaluated the ability of mPGES-1 siRNA to knock down LPS-induced mPGES-1 expression and PGE2 synthesis by RAW264.7 macrophages. As observed in Fig. 2A, LPS induced mPGES-1 expression in macrophages transfected with nonspecific siRNA. Paralleling the increase in mPGES-1 expression, levels of PGE2 in conditioned media recovered from LPS-treated cells were increased >50-fold compared with control cells (p < 0.005). Transfection with mPGES-1 siRNA effectively blocked LPS-induced mPGES-1 expression and reduced the induction of PGE2 levels by ~70% (p < 0.01). Next, we monitored MMP-induced MMP-9 expression in macrophages transfected with either nonspecific or mPGES-1 siRNA. MMP-9 mRNA was barely detected in control macrophages transfected with either nonspecific or mPGES-1 siRNA (Fig. 2B). Likewise, MMP-9 was barely detected in conditioned media derived from control cells transfected with either nonspecific or mPGES-1 siRNA (Fig. 2C). After incubation with MMP-1, MMP-3, or LPS, levels of MMP-9 mRNA and protein were markedly increased in macrophages transfected with nonspecific siRNA (p < 0.001; Fig. 2B, 2C). MMP-9 in conditioned medium recovered from MMP-3–treated macrophages was in the pro (105 kDa) and active (95 kDa) forms, confirming the ability of MMP-3 to activate pro–MMP-9 (34–37). Notably, both MMP- and LPS-induced MMP-9 expression was reduced ~70% in macrophages transfected with mPGES-1 siRNA (p < 0.001). Likewise, MMP-9 levels in conditioned media recovered from these cells were reduced ~65% (p < 0.001). Thus, mPGES-1 plays a principal role in proteinase and LPS-mediated induction of MMP-9.

**MMP-induced mPGES-1 expression is dependent on TNF-α**

Proteinases regulate cellular functions by activating protease-activated receptors (38, 39), releasing tethered growth factors (40–42) and cytokines (43, 44) or generating biologically active fragments of extracellular matrix (45–47). We previously reported that MMP-1– and MMP-3–induced MMP-9 expression in macrophages is dependent on the release of TNF-α, induction of Cox-2 expression, and PGE2 engagement of EP4 (17). Because mPGES-1 is induced in a variety of cells by inflammatory cyto-
kines, we determined whether MMP-mediated release of TNF-α from macrophages induced the expression of mPGES-1. First, we
examined the ability of recombinant murine TNF-α to induce
mPGES-1 in RAW264.7 macrophages. As observed in Fig. 3A,
levels of mPGES-1 mRNA increased, in a dose-dependent man-
ner, when cells were incubated with exogenous TNF-α (p < 0.05)
and reached levels achieved after incubation with LPS.

Second, we used a neutralizing rat monoclonal anti-murine
TNF-α IgG to determine whether MMP-induced release of
TNF-α was responsible for stimulating mPGES-1 expression in
macrophages. For this purpose, cells were preincubated 2 h with
20 μg/ml normal IgG or anti–TNF-α IgG prior to the addition of
MMPs or LPS. As shown in Fig. 3B, MMP-1 and MMP-3–mediated induction of mPGES-1 was blocked by anti–TNF-α IgG
(p < 0.05). Similarly, LPS-induced mPGES-1 expression was sup-
pressed ∼50% in the presence of anti–TNF-α IgG (p < 0.05). To
test directly the role of TNF-α in proteinase-induced mPGES-1,
macrophages were transfected with either nonspecific siRNA or
TNF-α siRNA as previously reported (17). The level of TNF-α
mRNA in cells transfected with TNF-α siRNA was reduced >80%
compared with that in cells transfected with nonspecific siRNA
(data not shown). When TNF-α siRNA transfected cells were ex-
posed to the metalloproteinases, MMP-1 and MMP-3 failed sig-
nificantly to induce mPGES-1 expression (p < 0.005). Moreover,
consistent with data obtained using anti–TNF-α IgG, TNF-α
knockdown attenuated LPS-induced mPGES-1 expression ∼50%
(p < 0.05) in transfected RAW264.7 macrophages (Fig. 3C).

**MMP-1 and MMP-3 induce MAPKerk1/2-dependent expression
of Egr-1**

The transcription factor Egr-1 plays a key role in regulating
mPGES-1 gene expression by binding to GC-box in the mPGES-1
promoter (48). To determine whether MMP-induced mPGES-1
expression was associated with changes in Egr-1 expression, mac-
rophages were treated with MMP-1 or MMP-3, and levels of
Egr-1 and mPGES-1 mRNA were monitored over time using PCR
(Fig. 4A). Levels of Egr-1 and mPGES-1 gene expression were
either undetectable or very low in control RAW264.7 macro-
phages. After the addition of MMPs, Egr-1 mRNA levels were
strongly elevated at 1 h and returned to low or undetectable levels
at 4 and 18 h. In contrast, levels of mPGES-1 mRNA were ele-
vated at 4 h and remained elevated over the experimental period
(18 h). Thus, MMP-induced Egr-1 expression preceded mPGES-1
expression. To determine whether MMP-dependent Egr-1 ex-
pression was dependent on the release of TNF-α, macrophages
were exposed to MMP-1 and MMP-3 in the presence of nonim-
mune IgG or anti–TNF-α IgG. As seen in Fig. 4B, anti–TNF-α
IgG reduced MMP-induced Egr-1 expression ∼65–80% in mac-
rophages (p < 0.01).

Because the induction of Egr-1 is regulated by MAPKerk1/2 (49–
51) and exposure of macrophages to MMP-1 and MMP-3 activates
MAPKerk1/2 (17), we determined whether MMP-induced Egr-1
and mPGES-1 expression was attenuated by an MEK-1 inhibitor
(U0126). Preincubation of macrophages with U0126 blocked
MMP-induced Egr-1 expression (Fig. 5A) and mPGES-1 expres-
sion (Fig. 5B) >90% (p < 0.001). Together with results of our
earlier studies (17), these data indicate that MMP-dependent re-
lease of TNF-α triggers phosphorylation of MAPKerk1/2, induction
of Egr-1, and subsequently mPGES-1.

**EP4-dependent positive feedback loop regulates mPGES-1
expression**

The diverse physiological and pathophysiological effects of PGE2
are mediated by the EP family of prostanoid receptors (52, 53).
The EP family is comprised of four subtypes (EP 1–4), which
are mediated by the EP family of prostanoid receptors (52, 53).

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![FIGURE 2](http://www.jimmunol.org/)
TNF-α, or LPS (data not shown) and their ability to induce mPGES-1 expression (p < 0.001; Fig. 6B).

Next, we examined whether MMP-induced mPGES-1 expression was dependent on PGE2 engagement of EP4. In this regard, we determined whether macrophage expression of the PGE2 receptor family members (i.e., EP 1–4) was regulated by MMPs. We previously reported that RAW264.7 macrophages constitutively express EP1–4 (29). After 18-h incubation with MMP-1 and MMP-3, levels of EP2 and EP4 mRNA were elevated (Fig. 7A), whereas there were no changes in expression of EP1 and EP3 (data not shown). LPS-treated macrophages serve as a positive control in these studies (54, 55). To differentiate the roles of EP2 and EP4 in mPGES-1 expression, cells were preincubated 30 min with antagonists of EP2 (AH6809) or EP4 (AH23848) prior to incubation with LPS (Fig. 7B). Over a range of 0.1–10 μM, the EP2 antagonist had no effect on LPS-induced mPGES-1 expression, whereas LPS-induced mPGES-1 expression was attenuated by 65% and 90% with 5 μM and 10 μM EP4 antagonist, respectively (p < 0.05). Likewise, preincubation with the EP4 antagonist reduced MMP-1– and MMP-3–induced mPGES-1 expression by >90% and 70%, respectively (p < 0.005), whereas the EP2 antagonist had no effect (Fig. 7C).

FIGURE 3. Neutralizing anti–TNF-α IgG and TNF-α silencing blocks MMP-induced mPGES-1 expression. A, RAW264.7 macrophages (6-well plate; 2 × 10⁶ cells/well) were incubated 18 h in DMEM–0.1% LE-BSA containing 0–100 ng/ml recombinant murine TNF-α. Total RNA was recovered, and levels of mPGES-1 mRNA were determined using real-time PCR. B, Macrophages (6-well plate; 2 × 10⁶/well) were preincubated 2 h with nonimmune IgG1 (nIgG; 20 μg/ml) or rat monoclonal anti-mouse TNF-α IgG1 (20 μg/ml), followed by 18-h incubation with 50 nM MMP-1 or MMP-3 or 10 ng/ml LPS. C, Macrophages (6-well plate; 2 × 10⁶/well) transfected with nonspecific (NS) or TNF-α siRNA were incubated 18 h with DMEM–0.1% LE-BSA (Ctrl) or media containing MMP-1, MMP-3, or LPS. Total RNA was recovered, and levels of mPGES-1 and actin mRNA were determined using PCR. Data are representative blots and the mean levels (± SD) of mPGES-1 mRNA, presented as RDUs, of three experiments.

FIGURE 4. MMP-induced Egr-1 is dependent on TNF-α. A, RAW264.7 macrophages (12-well plate; 7 × 10⁵/well) were incubated 0–18 h in DMEM–0.1% LE-BSA (Ctrl) or media containing 50 nM MMP-1 or MMP-3. B, In other experiments, macrophages (6-well plate; 2 × 10⁶/well) were preincubated for 2 h with nonimmune IgG1 (nIgG; 20 μg/ml) or rat monoclonal anti-mouse TNF-α IgG1 (20 μg/ml), followed by 1-h incubation with 50 nM MMP-1 or MMP-3. Total RNA was isolated, and mRNA levels for Egr-1, mPGES-1, and actin were determined by PCR. Data are representative blots and the mean levels (± SD) of target mRNAs, presented as RDUs, of three experiments.
To test directly whether PGE$_2$ binding to EP4 underlies a feedback loop (EP4 → MAPK$_{erk1/2}$ → Egr-1) that regulates mPGES-1 expression, we first examined the abilities of exogenous PGE$_2$, EP2 agonist butaprost (56), and EP4 agonist PGE$_1$-OH (57) to trigger phosphorylation of MAPK$_{erk1/2}$ (Fig. 8A). Treatment of cells with 10$\mu$M PGE$_2$ or PGE$_1$-OH for 0–25 min resulted in a rapid increase in phosphorylation of MAPK$_{erk1/2}$ ($p<0.01$ and $p<0.02$, respectively). In contrast, incubation with butaprost had no significant effect. These data corroborate earlier reports demonstrating that PGE$_2$ → EP4 → MAPK$_{erk1/2}$ in HEK-293 cells transfected with EP4 (51) and HCA-7 colon cancer cells (50). Having confirmed that PGE$_2$ → EP4 → MAPK$_{erk1/2}$, we next determined the ability of an EP4 antagonist to block PGE$_2$-induced Egr-1 and mPGES-1 expression in macrophages. As observed in Fig. 8B, preincubation of macrophages with EP4 antagonist AH23848 blocked PGE$_2$-induced Egr-1 and mPGES-1 expression ($p<0.01$). Similarly, preincubation of macrophages with ONO-AE3-208, another EP4 antagonist, also blocked PGE$_2$-induced Egr-1 and mPGES-1 expression ($p<0.01$). Thus, MMP induction of Cox-2 and subsequent PGE$_2$ secretion contributes to an EP4-dependent feedback loop that regulates mPGES-1 expression in macrophages.

Finally, we used a genetic approach to interrogate the role of EP4 in mPGES-1 expression. For this purpose, RAW264.7 macrophages were transfected with either nonspecific siRNA or EP4 siRNA and exposed to LPS. As expected, LPS potently induced Cox-2 and mPGES-1 expression in RAW264.7 macrophages transfected with nonspecific siRNA (Fig. 9A) and led to >900% increase in the level of PGE$_2$ in their conditioned media (Fig. 9B). In contrast, LPS-induced mPGES-1 ($p<0.04$) and Cox-2 ($p<0.01$) expression was markedly attenuated in macrophages transfected with EP4 siRNA, which resulted in approximately a 75% reduction in LPS-mediated induction of PGE$_2$ levels.

**EP4 antagonist attenuates mPGES-1 expression in thioglycolate-elicited macrophages**

Results of experiments described here reveal that PGE$_2$ engagement of EP4 regulates the major inducible form of PGE synthase (i.e., mPGES-1) in RAW264.7 macrophages. Thus, the administration of an EP4 antagonist to inhibit macrophage MMP-9 expression (29) would be expected to drive down levels of PGE$_2$ by attenuating both Cox-2 and mPGES-1 expression. To test this hypothesis, macrophages recovered from thioglycolate-induced
peritonitis were pretreated with AH23848 or celecoxib followed by an 18-h incubation with LPS. As shown in Fig. 10A, LPS potently induced Cox-2 and mPGES-1 expression. Commensurate with the observed increase in Cox-2 and mPGES-1 expression, the levels of PGE2 in conditioned media recovered from these cells was increased ∼5-fold (p < 0.005) compared with untreated macrophages (Fig. 10B). Pretreatment with either AH23848 or celecoxib attenuated LPS-induced Cox-2 expression (∼80%; p < 0.001). Likewise, AH23848 and celecoxib similarly reduced LPS-induced Cox-2 expression (∼50%; p < 0.05) (Fig. 10A). However, the effect of the EP4 antagonist and Cox-2 inhibitor on PGE2 levels in macrophage conditioned media was significantly different. Treatment with celecoxib reduced LPS-induced levels of PGE2 to those observed in untreated control cells. In contrast, although treatment with AH23848 reduced LPS-induced levels of PGE2 to 60% (p < 0.005), they remained elevated relative to controls (p < 0.05). This reflects the ability of celecoxib to both inhibit Cox-2 activity and attenuate Cox-2 and mPGES-1 expression. Finally, both treatments were effective in blocking LPS-induced MMP-9 expression by thioglycolate-elicited macrophages (Fig. 10C). Taken together, these data confirm that administration of an EP4 antagonist to inhibit macrophage MMP-9 expression results in reduced PGE2 synthesis by attenuating both Cox-2 and mPGES-1 expression (Fig. 11).

Discussion

MMP-9 plays a significant role in the pathogenesis of chronic inflammatory diseases and cancer (10–16). Thus, identifying targetable components of signaling pathways that regulate MMP-9 expression may have broad therapeutic implications. Earlier, we reported that MMP-1 and MMP-3 stimulate macrophage MMP-9 expression via the release of TNF-α and subsequent induction of Cox-2 and PGE2 engagement of EP4 receptor (17). Although it is well established that Cox activity plays a major role in the synthesis of PGE2 under normal and pathological settings, the isomerization of Cox-derived PGH2 to PGE2 is catalyzed by a family of PGE synthases (18, 19). In this study, the regulatory role MMPs may have on the expression of PGE synthases was examined. We found that MMP-1- and MMP-3-dependent release of TNF-α induced rapid and transient expression of Egr-1 in macrophages followed by sustained elevation in mPGES-1 expression. Moreover, proteinase-induced MMP-9 expression was markedly attenuated in macrophages in which mPGES-1 was silenced. Thus, inhibition of mPGES-1 expression and/or activity emerges as a therapeutic target in the regulation of MMP-9 expression (Fig. 11).

Elevated levels of PGE2 associated with inflammation and several cancers depends on the coupling of Cox-2 and mPGES-1 expression (58–62) (23, 32, 63). MMP-mediated induction of Cox-2 and mPGES-1 in macrophages suggests the activation of a common signaling pathway. In this regard, we previously have shown that the activation of MAPKerk1/2 is a key determinant of MMP-induced Cox-2 expression and MMP-9 synthesis (17). The expression of Egr-1, a transcription factor that recognizes GC-rich sequences in the mPGES-1 promoter (18), is dependent on MAPKerk1/2 signaling (50, 51). Therefore, we examined whether proteinase triggered activation of MAPKerk1/2 played a role in...
Egr-1 and mPGES-1 expression. Our studies demonstrate that MMP-induced Egr-1 gene expression was rapid and transient and preceded mPGES-1 expression. Importantly, preincubation of macrophages with a MEK-1 inhibitor blocked MMP-1 and MMP-3 induction of mPGES-1. Thus, MMP-triggered activation of MAPKerk1/2 appears to be the common signaling pathway that drives MMP-induced Cox-2 and mPGES-1 expression in macrophages (Fig. 11).

The diverse biologic actions of PGE2 are mediated by the EP1–4 family of prostanoid receptors (52, 53). In this study, we report that MMP-1 and MMP-3 induce macrophage EP2 and EP4 expression. We and others have demonstrated that PGE2 engagement of EP4 stimulates macrophage MMP-9 expression (29, 64) and enhances Cox-2 expression via a positive feedback loop (29, 65, 66). It has been reported that PGE2 → EP4 triggers MAPKerk1/2-dependent expression of Egr-1 (50, 51, 67). As reported in this study, PGE2 and the selective EP4 agonist PGE1-OH triggered rapid and transient phosphorylation of MAPK erk1/2 in macrophages; and PGE2-induced Egr-1 and mPGES-1 expression was blocked by the EP4 antagonists AH23848 and ONO-AE3-208. Thus, PGE2 → EP4 participates in a feedback loop driving mPGES-1 expression. This pathway was shown to be critical in MMP-induced mPGES-1 expression inasmuch that MMP-triggered MAPK erk1/2-dependent induction of Egr-1 and mPGES-1 was blocked when macrophages were incubated with either celecoxib or an EP4 antagonist (Fig. 11).

Our studies reveal a nexus between proteinases and prostanoids whereby MMP-1 and MMP-3, proteinases commonly found in inflammatory foci, stimulate macrophage secretion of PGE2 by inducing Cox-2 and mPGES-1 expression. PGE2 is a major member of the prostanoid class of bioactive lipids with diverse immunomodulatory functions (68, 69). The traditional view of PGE2 as an immunosuppressant is, in part, derived from its ability to inhibit T cell proliferation and differentially regulate Th1 and Th2 cytokine expression (70, 71) and to attenuate cytokine and chemokine expression by inflammatory macrophages (72–74). Moreover, results of recent experiments suggest that PGE2 engagement of EP4 suppresses inflammation in vivo. The conditional deletion of EP4 in macrophages and/or microglia increased proinflammatory gene expression in brain and isolated microglia...
after LPS administration (75). Similarly, deletion of EP4 in bone marrow-derived cells enhanced inflammation associated with experimental models of atherosclerosis (76) and aneurysm formation (77), and administration of EP4 agonist suppressed production of proinflammatory cytokines and chemokines in cardiac transplants (78).

Despite the reported immunosuppressive and anti-inflammatory actions of PGE2, several lines of evidence support a critical role of mPGES-1-derived PGE2 in the pathogenesis of chronic inflammatory diseases. For example, PGE2 stimulates edema formation (24), pain (24, 79), and proteinase expression (80–83). In addition, thioglycolate-induced recruitment of macrophages to the peritoneal cavity and carrageenan-induced leukocyte recruitment to the pleural cavity are attenuated in mPGES-1 null mice (79, 84). Finally, the deletion of mPGES-1 markedly attenuates experimentally induced arthritis (24, 79), atherosclerosis (85), and aneurysm formation (86). Moreover, PGE2 acting via EP4 on T cells and dendritic cells facilitates Th1 cell differentiation and amplifies IL-23–mediated Th17 cell expansion in vitro (87). Administration of EP4 antagonists decreases accumulation of both Th1 and Th17 cells in regional lymph nodes and suppresses experimentally induced autoimmune encephalomyelitis and contact hypersensitivity (87). Likewise, administration of EP4 antagonists inhibits inflammation in experimental models of skin injury (88) and arthritis (89). Similarly, experimentally induced arthritis was reduced in EP4 null mice (90).

Clearly, understanding the environmental context that determines whether PGE2 acts in an immunosuppressive/anti-inflammatory or immunostimulatory/proinflammatory capacity remains a critical goal. We have reported that MMP-1 and MMP-3 stimulate macrophage MMP-9 expression by releasing TNF-α, which upregulates key components of the PGE2 biosynthetic and signaling pathways (i.e., Cox-2, mPGES-1, and EP4) (i.e., Cox-2, mPGES-1, and EP4). Proteinase-induced PGE2 synthesis and EP4 expression likely contribute to the inflammatory response, as PGE2 engagement of EP4 stimulates MMP-9 expression, which is causally associated with the pathogenesis of several inflammatory diseases. Selective targeting of EP4 blocks PGE2-dependent MMP-9 expression and attenuates PGE2 synthesis by eliminating a feedback loop attenuating expression of Cox-2 and mPGES-1.

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Disclosures
A.J.D. is a member of the Scientific Advisory Board of Tragara Pharma- ceuticals, Inc., a company that is developing a selective COX-2 inhibitor. The other authors have no financial conflicts of interest.

References


Corrections


The mPGES-1 PCR blots in Fig. 8C were inadvertently duplicated from the Egr-1 blots due to an error in figure preparation. The corrected version of Fig. 8 is shown below. The figure legend was correct as published and is shown below for reference.
**FIGURE 8.** PGE₂ binding to EP4 triggers the activation of MAPK\(_{\text{erk}1/2}\) and Egr-1 and mPGES-1 expression. A, RAW264.7 macrophages (12-well plate; 5 × 10⁵/well) were cultured in DMEM–0.1% LE-BSA for 24 h. Cells were then incubated with 10 μM butaprost (EP2 agonist), PGE₂, or PGE₁-OH (EP4 agonist) for 0–25 min, and lysates were prepared. Levels of phosphorylated and total MAPK\(_{\text{erk}1/2}\) were determined using Western blot. Data are representative blots and the mean levels (±SD) of phosphorylated MAPK\(_{\text{erk}1/2}\) ("P-\text{erk}1/2"), presented as RDUs, of three experiments. B and C, Macrophages (12-well plate; 5 × 10⁵ to 7 × 10⁵/well) were preincubated 30 min in DMEM–0.1% LE-BSA or media containing EP4 antagonists AH23848 or ONO-AE2-208, followed by PGE₂. Total RNA was collected at 30 min and 18 h, and mRNA levels for Egr-1, mPGES-1, and actin were determined using PCR. Data are representative blots and the mean levels (±SD) of target mRNAs, presented as RDUs, of three experiments.