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Shunting of prostanoid biosynthesis in microsomal prostaglandin E synthase-1 null embryo fibroblasts: regulatory effects on inducible nitric oxide synthase expression and nitrite synthesis

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Abstract

Microsomal prostaglandin (PG) E synthase (mPGES)-1 is an inducible enzyme that acts downstream of cyclooxygenase (COX) and specifically catalyzes the conversion of prostaglandin (PG)H₂ to PGE₂, most prominently in inflammatory conditions. Specific inhibitors of mPGES-1 are not yet available, however, mice with genetic deletion of mPGES-1 have been generated that have given insight into the specific role of mPGES-1 in eicosanoid biosynthesis *in vivo* and in peritoneal macrophages. We created mouse embryo fibroblast (MEF) cell lines that would facilitate investigation of the effect of mPGES-1 genetic deletion on prostanoid biosynthesis in fibroblast lineage cells and its subsequent effect on the expression of inducible NOS (iNOS) and nitrite biosynthesis using cells derived from mPGES-1 wild-type (WT), heterozygous (Het), and null mice. The results show that genetic deletion of mPGES-1 results in a dramatic decrease in PGE₂ production in Het and null MEFs under basal conditions and after stimulation with interleukin (IL)-1 β , suggesting that mPGES-1 is critically important for PGE₂ production. Furthermore, we show that mPGES-1 gene deletion results in diversion of prostanoid production from PGE₂ to 6-keto PGF_{1 α} (the stable metabolic product of PGI₂; prostacyclin) in a gene dose-dependent manner in Het and null MEFs compared with their WT counterparts, suggesting a shunting phenomenon within the arachidonic acid (AA) metabolic pathway. In addition, we show that mPGES-1 gene deletion and subsequent decrease in PGE₂ levels results in a differential induction profile of iNOS and nitrite levels (the stable breakdown product of nitric oxide (NO) in mPGES-1 WT MEFs compared with null MEFs. These results provide important information regarding the therapeutic potential for pharmacologic inhibition of mPGES-1 in inflammatory conditions.—Kapoor, M., Kojima, F., Qian, M., Yang, L., and Crofford, L. J. Shunting of prostanoid biosynthesis in microsomal prostaglandin E synthase-1 null embryo fibroblasts: regulatory effects on inducible nitric oxide synthase expression and nitrite synthesis.

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Keywords

arachidonic acid (AA); cyclooxygenase (COX); L-arginine

During an inflammatory insult, one of the essential fatty acids released by cell membrane phospholipids is AA, which is subsequently metabolized by a series of enzymatic reactions to yield biologically active mediators. COX-1 and -2 convert AA into PGH₂, which is further metabolized by terminal PG synthases to PGE₂, 6-keto PGF_{1α}, PGD₂, PGF_{2α}, and thromboxane (TX) A₂.

Among these prostanoids, PGE₂ is a key mediator of inflammation. Nonsteroidal antiinflammatory drugs (NSAIDs), including COX-2-selective NSAIDs, mediate their antiinflammatory effects by inhibiting the inducible COX-2 enzyme and decreasing the production of PGE₂ at inflammatory sites (1–3). However, gastrointestinal, renal, and the recently reported cardiovascular side effects associated with the pharmacological inhibition of the COX enzymes (4–8) have led to renewed attention to other potential targets for antiinflammatory drugs. One of the most promising of these is the inducible form of PGE synthase, which acts downstream of COX enzymes and specifically converts PGH₂ to PGE₂ after an inflammatory insult (9–12).

Three PGE synthases, including cytosolic PGE synthase (cPGES), mPGES-1, and mPGES-2, have been cloned and characterized (9, 11, 12). cPGES is localized in the cytosolic region of a variety of cells and tissues under basal conditions and is functionally coupled with COX-1. cPGES is most likely to be involved in the production of PGE₂ for the maintenance of homeostasis (11). mPGES-2 is also constitutively expressed in wide variety of tissues and cell types and is synthesized as a golgi membrane-associated protein (13). There appears to be no differential coupling of mPGES-2 with the COX enzymes. In contrast, mPGES-1 is an inducible enzyme that acts downstream of COX and specifically catalyzes the conversion of PGH₂ to PGE₂ (9), most prominently in inflammatory conditions (14, 15). Based on observations in mice genetically deficient in mPGES-1, this enzyme is a critically important mediator of pain, angiogenesis, fever, bone metabolism, and tumorigenesis (16–19).

We have previously shown that mPGES-1 expression is increased in tissues and cells of various inflammatory conditions, including rheumatoid arthritis (RA) and osteoarthritis (OA) (14, 15, 20, 21). In addition, we have also shown that mPGES-1 is essential for increased PGE₂ production. Furthermore, there are reports that mPGES-1 null mice are resistant to chronic inflammation of joints in the models of collagen-induced arthritis (CIA) and collagen antibody (Ab)-induced arthritis (CAIA) (16, 17).

NSAIDs, including COX-2-selective inhibitors, are used extensively to treat arthritis and other inflammatory conditions. However, by inhibiting COX enzymes, these drugs not only inhibit the production of the major proinflammatory prostaglandin PGE₂, but they also block the synthesis of other prostanoids involved in maintaining key physiological functions of a cell/tissue. To overcome the limitations/side effects associated with the inhibition of COX enzymes, mPGES-1 is an attractive target to achieve more specific inhibition of PGE₂

production associated with inflammatory disorders. However, it is critically important in preclinical work to understand the potential consequences of inhibiting this enzyme.

In the present study we demonstrate the consequences of mPGES-1 genetic deletion on prostanoid biosynthesis using mPGES-1 null mice-derived embryo fibroblasts. The results show that genetic deletion of mPGES-1 results in a dramatic decrease in PGE₂ production in mPGES-1 Het and null MEFs under basal and proinflammatory cytokine stimulated conditions, suggesting that mPGES-1 is critically important for PGE₂ production. This study further demonstrates higher levels of 6-keto PGF_{1α} in mPGES-1 Het and null MEFs compared with their WT counterparts, suggesting a shunting phenomenon within the AA metabolic pathway associated with mPGES-1 deletion in a gene dose-dependent manner. Furthermore, we show that mPGES-1 gene deletion and resultant decrease in PGE₂ production results in the elevation of iNOS expression and nitrite levels in mPGES-1 null MEFs compared with WT MEFs, suggesting that effects of mPGES-1 deletion may not be restricted to differential production of prostanoids within the AA metabolic pathway but can affect the L-arginine metabolic pathway involving iNOS.

MATERIALS AND METHODS

Animals

mPGES-1 null, Het, and littermate WT mice (DBA1 lac/J background) were obtained from Pfizer Inc (Groton, PA, USA) (17). COX-2 null and littermate WT mice (C57BL/6 background) were obtained from Robert Langenbach (National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA) (22). Mice were housed in microisolator cages in a pathogen-free barrier facility, and all experiments were performed under the approved IACUC guidelines.

Materials

Rabbit anti-human mPGES-1 antiserum was gifted from Dr. Per-Johan Jakobsson (Karolinska Institute, Stockholm, Sweden). Rabbit anti-mouse COX-2 polyclonal antibody (pAb), rabbit anti-human mPGES-2 pAb, rabbit anti-human cPGES pAb, rabbit anti-mouse PGI synthase (PGIS) pAb, rabbit anti-mouse hematopoietic PGD synthase (H-PGDS) pAb, rabbit anti-human TX synthase (TXS) pAb, rabbit anti-mouse COX-1 pAb, PGE₂, NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide), ovine COX-2 standard protein and ELISA kits for PGE₂, 6-keto PGF_{1α}, PGD₂ and TXB₂ were all purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Rabbit anti-human eNOS pAb and rabbit anti-human iNOS pAb were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-human beta-actin monoclonal antibody (mAb) and indomethacin were obtained from Sigma Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Dulbecco's modified Eagle medium (DMEM) and FBS were from Invitrogen (Carlsbad, CA, USA). Recombinant mouse IL-1β was obtained from R&D Systems (Minneapolis, MN, USA). TRIPure was purchased from Roche Diagnostics (Indianapolis, IN, USA). The polyvinylidene difluoride (PVDF) membrane and

enhanced chemiluminescence (ECL) reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Preparation and activation of mouse embryo fibroblasts

Embryos were harvested from mPGES-1 (DBA1 lac/J) or COX-2 (C57 BL/6) pregnant Het females (E12.5) who had been mated with Het males. Whole embryos were mixed and placed into culture DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C under an atmosphere of 5% CO₂. At confluence, the cells were detached and passaged and the 3–4 passage cells were used in the following experiments. MEFs were plated into the wells of a 6-well plate at a density of 3×10^5 cells/well in DMEM containing 10% FBS. Cells were starved for 72 h in DMEM containing 1% FBS and then incubated with or without 1 ng/ml IL-1β. Cell viability was determined by measuring mitochondrial NADH-dependent dehydrogenase activity with WST-1 assay (Dojindo Laboratories, Kumamoto, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA from the cells was extracted with TRIpure reagent according to the manufacturer's instructions. Reverse transcription was performed according to the manufacturer's instructions using a SuperScript preamplification system (Invitrogen, Carlsbad, CA, USA) with 1 µg of total RNA from the cells as a template. Subsequent amplifications of the cDNA fragments by PCR with HotStarTaq polymerase (Qiagen, Valencia, CA, USA) were performed using 0.5 µl of the reverse-transcribed mixture as a template with specific oligonucleotide primers and cycle number as follows: mouse mPGES-1 (28 cycles), sense 5'-CAC ACT GCT GGT CAT CAA GA-3', and antisense 5'-ACA CCA AGT CCG CAA GTT C-3'; mouse COX-2 (25 cycles), sense 5'-GGG CCC TTC CTC CAG TAG CAG A-3', and antisense 5'-CAG ACC AGG CAC CAG ACC AA-3'; mouse mPGES-2 (25 cycles), sense 5'-GGT GGC CCA GGA AGG AGA CAG C-3', and antisense 5'-GCA GCC GCG CCC ACA TAC TTG-3'; mouse cPGES (30 cycles), sense 5'-CCC GCC CAC CCG TTT GTC-3', and antisense 5'-TCT GGC ATC TTT TCA TCA TCA CTG-3'; mouse PGIS (30 cycles), sense 5'-TTC TGG CTC CTT CTT TTC CTC CTC-3', and antisense 5'-CTT CAG CCG TTT CCC ATC TTT GTA -3'; mouse H-PGDS (31 cycles), sense 5'-ATG CCT AAC TAC AAA CTG CTT-3', and antisense 5'-CTA GAG TTT TGT CTG TGG CCT-3'; mouse TXS (30 cycles), sense 5'-CCT CTG TGT GGG TAC TAT CT-3', and antisense 5'-TGG GAC CAT TAT GGA TGG AA-3'; mouse COX-1 (30 cycles), sense 5'-CCC CAG CCC TCC GAC CTA CAA-3' and antisense 5'-CCC CGG AAG CAA CCC AAA CAC-3'; mouse GAPDH (20 cycles), sense 5'-GGG GTG AGG CCG GTG CTG AGT AT-3', and antisense 5'-TGG GGG TAG GAA CAC GGA AGG-3'. After initial denaturation at 95°C for 15 min, PCR involved amplification cycles of 30 s at 95°C, 30 s at 56°C, and 45 s at 72°C, followed by elongation for 5 min at 72°C. The amplified cDNA fragments were resolved by electrophoresis on 2% (w/v) agarose gel and were visualized under UV light using a Bio-Rad Chemidoc Apparatus (Hercules, CA, USA) after staining of the gel with ethidium bromide.

Western blotting

Cells were lysed in Tris-buffered saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS), and the protein content of the lysates was determined using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA) with BSA as the standard. Cell lysates were adjusted to equal equivalents of protein and then were applied to SDS-polyacrylamide gels (10–20%) for electrophoresis. Next, the proteins were electroblotted onto VDF membranes. After the membranes were blocked in 10 mM TBS containing 0.1% Tween-20 (TBS-T) and 5% skim milk, the membranes were probed for 1.5 h with the respective antibodies (1:500 for mPGES-1, COX-2, mPGES-2, cPGES, PGIS, H-PGDS, TXS, COX-1, eNOS, and iNOS; 1:1000 for beta-actin) in TBS-T for 1.5 h. After washing the membranes with TBS-T, the membranes were incubated with HRP-conjugated anti-rabbit (for mPGES-1, COX-2, mPGES-2, cPGES, PGIS, H-PGDS, TXS, COX-1, eNOS, and iNOS) or HRP-conjugated anti-mouse (for beta-actin) IgG (1:10,000 dilution in TBS-T containing 5% skim milk) for overnight at 4°C. After further washing with TBS-T, protein bands were visualized with an ECL Western blot analysis system using a Bio-Rad Chemidoc Apparatus (Bio-Rad, Hercules, CA, USA).

Measurement of prostaglandins in culture medium

MEFs were incubated for 12 h in the presence or absence of IL-1 β (1 ng/ml). In experiments involving treatment with indomethacin and NS-398, these compounds were added 72 h before IL-1 β stimulation. The culture supernatant was harvested and the concentrations of PGE₂, 6-keto PGF_{1 α} (a stable metabolite of PGI₂), PGD₂, and TXB₂ (a stable metabolite of TXA₂) were measured by ELISA. Assays were performed according to the manufacturer's recommendation.

Measurement of nitrite in culture medium

For measurement of nitrite in the cell supernatants, MEFs were plated into the wells of a 6-well plate at a density of 3×10^5 cells/well in phenol red free DMEM containing 10% FBS. Cells were starved for 72 h in phenol red free DMEM containing 1% FBS and then incubated for 12 h in the presence or absence of IL-1 β (1 ng/ml), with or without indomethacin, NS-398, and PGE₂. Nitrite concentrations were measured in cell supernatants as an indicator of NO production using a commercially available Griess reaction kit (Cayman, Ann Arbor, MI, USA). Assay was performed according to the manufacturer's recommendation.

Statistical analysis

The data are expressed as mean $\pm$ SEM. Statistical analysis was done using two-way ANOVA followed by Tukey test. $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of mPGES-1 WT, Het, and null MEFs

We first characterized the genotype of mPGES-1 MEFs using RT-PCR. Fig 1A shows the mRNA expressions for mPGES-1 WT, Het, and null MEFs. In addition, we also assessed

the cell viability of mPGES-1 WT, Het, and null MEFs with or without IL-1 β stimulation using WST-1 cell viability assay. No change in the cell viability of mPGES-1 WT, Het, and null MEFs was observed with or without IL-1 β stimulation (data not shown).

mRNA expression profile of prostanoid biosynthetic enzymes in mPGES-1 WT and null MEFs

Incubation of mPGES-1 WT and null MEFs with IL-1 β resulted in an up-regulation of mPGES-1 mRNA expression in mPGES-1 WT MEFs, with peak expression levels observed at 3 h post IL-1 β stimulation (Fig 1B), while no band was detected in mPGES-1 null MEFs. COX-2 mRNA was coinduced with mPGES-1 mRNA in both WT and null MEFs. However, the peak mRNA expression of COX-2 was observed at 1 h post IL-1 β stimulation in both WT and null MEFs, earlier to that of mPGES-1 expression. In addition, we also observed that IL-1 β induced COX-2 mRNA expression in mPGES-1 null MEFs was higher compared with their WT counterparts throughout the time course of 24 h.

We also determined whether mPGES-1 WT and null MEFs expressed other prostanoid biosynthetic enzymes, including mPGES-2, cPGES, PGIS, H-PGDS, TXS, and COX-1. Results showed that mPGES-2, cPGES, PGIS, H-PGDS, TXS, and COX-1 were constitutively expressed in WT and null MEFs as no significant change in the mRNA expression levels of these enzymes was observed throughout the time course of 24 h, post IL-1 β stimulation. We did not observe differences in expression of these enzymes comparing mPGES-1 WT and null MEFs. (Fig 1B).

Protein expression profile of prostanoid biosynthetic enzymes in mPGES-1 WT and null MEFs

Incubation of mPGES-1 WT and null MEFs with IL-1 β resulted in an up-regulation of mPGES-1 protein expression in mPGES-1 WT MEFs, with peak expression levels observed at 48 h post IL-1 β stimulation (Fig. 2). COX-2 protein expression was coinduced with mPGES-1 in both WT and null MEFs. However, the peak protein expression of COX-2 was observed at 12 h post IL-1 β stimulation in both WT and null MEFs, earlier than that observed for mPGES-1 protein expression. The protein expression profile of COX-2 and mPGES-1 correlated to that observed at mRNA level. However, we observed that IL-1 β induced COX-2 protein expression in mPGES-1 WT MEFs was higher compared with their null counterparts throughout the time course of 48 h, opposite to what we observed for COX-2 mRNA expression (Fig 1B).

The protein expression profile of other prostanoid biosynthetic enzymes including mPGES-2, cPGES, PGIS, H-PGDS, TXS, and COX-1 mirrored that of mRNA levels. All enzymes were constitutively expressed in WT and null MEFs, as no significant change in the protein expression levels of these enzymes was observed throughout the time course of 48 h, post IL-1 β stimulation (Fig. 2). No differences in protein expression of these enzymes were observed in mPGES-1 WT MEFs compared to null MEFs.

Differential pattern of endogenous prostanoid production in mPGES-1 WT, Het, and null MEFs

Higher levels of endogenous PGE₂ were observed in mPGES-1 WT MEFs compared with significantly lower ($P<0.05$) baseline levels observed in mPGES-1 null counterparts under unstimulated basal condition (Fig. 3A). On stimulation with IL-1 β , a significant increase ($P<0.01$) in the levels of PGE₂ was observed in the mPGES-1 WT MEFs, at 6, 12, and 24 h, peaking at 12 h post IL-1 β stimulation and correlating with the peak expression profile of COX-2 protein as shown in Fig. 2. However, IL-1 β stimulation did not show any significant change in the PGE₂ levels in the mPGES-1 null MEFs and the levels were significantly lower ($P<0.05$ at 1 h; $P<0.01$ at 3, 6, 12, 24 h) compared with their IL-1 β stimulated WT counterparts even though COX-2 protein was expressed. These results clearly suggest that mPGES-1 is vital for PGE₂ production under basal conditions but almost essential in conjunction with COX-2 for PGE₂ production during inflammatory conditions.

We further determined the levels of endogenous 6-keto PGF_{1 α} in mPGES-1 WT and null MEFs. The MEFs synthesize high levels of 6-keto PGF_{1 α} , with 5-fold higher 6-keto PGF_{1 α} levels compared to that of PGE₂. The levels of 6-keto PGF_{1 α} in mPGES-1 WT and null MEFs showed a significant difference under normal basal conditions with significantly higher levels ($P<0.05$) of 6-keto PGF_{1 α} observed in mPGES-1 null MEFs. Stimulation with IL-1 β resulted in a significant increase ($P<0.01$) in the levels of 6-keto PGF_{1 α} in mPGES-1 null MEFs, at 1, 3, 6, 12, and 24 h, peaking at 6–12 h post IL-1 β stimulation. In contrast, stimulation with IL-1 β exhibited almost negligible up-regulation in the levels of 6-keto PGF_{1 α} in mPGES-1 WT MEFs compared with mPGES-1 null MEFs (Fig 3A). Levels of PGD₂ and TXB₂ were also assessed; however, no significant change in the levels of PGD₂ and TXB₂ was observed with and without IL-1 β stimulation at any time points in mPGES-1 WT MEFs and null MEFs (Fig 3A).

These results suggest that genetic deletion of mPGES-1 shifts prostanoid production profile from PGE₂ to 6-keto PGF_{1 α} in cells where these are the dominant PG products. To further confirm the differential shift in the prostanoid production, we determined the levels of PGE₂ and 6-keto PGF_{1 α} in IL-1 β stimulated and unstimulated mPGES-1 WT, Het, and null MEFs. The results clearly suggest that deletion of mPGES-1 gene directly results in the up-regulation of 6-keto PGF_{1 α} levels in mPGES-1 Het and null MEFs vs. PGE₂ production in mPGES-1 WT MEFs in a gene dose-dependent manner (Fig 3B).

Effect of nonselective and selective COX-2 inhibition on the levels of PGE₂ and 6-keto PGF_{1 α} in mPGES-1 WT and null MEFs

To evaluate the contribution of COX-2 (upstream enzyme for mPGES-1) toward the production of PGE₂ and 6-keto PGF_{1 α} , we determined the effects of the nonselective COX inhibitor, indomethacin, and the selective COX-2 inhibitor, NS-398, on the prostanoid production profile. Treatment with indomethacin and NS-398 significantly inhibited the production of both PGE₂ and 6-keto PGF_{1 α} in IL-1 β stimulated and unstimulated WT and null MEFs (Fig. 4). Since indomethacin and NS-398 showed similar extent of prostanoid inhibition, these results suggest that COX-2, and not COX-1, is the main upstream enzyme responsible for the production of PGE₂ and 6-keto PGF_{1 α} in MEFs.

Effect of genetic deletion of COX-2 on the levels of PGE₂ and 6-keto PGF_{1α} in COX-2 WT, Het, and null MEFs

To further evaluate the contribution of COX-2 toward the production of PGE₂ and 6-keto PGF_{1α}, we assessed the levels of these prostanoids in the MEFs isolated from mice with a genetically deleted COX-2 gene (Fig 5A). The levels of PGE₂ and 6-keto PGF_{1α} observed in the COX-2 WT MEFs (isolated from mouse on C57BL/6 background) were almost similar to what we observed for mPGES-1 WT MEFs (isolated from mice on DBA1/lacJ background), including higher absolute levels of 6-keto PGF_{1α} than PGE₂ (Fig 5B). Thus, using MEFs from two different background strains did not result in a dramatic change in the levels of these prostanoids.

Stimulation with IL-1β resulted in a significant increase in the levels of PGE₂ in the COX-2 WT MEFs (Fig 5B). However, IL-1β stimulation had no effect on the levels of PGE₂ in COX-2 null MEFs, which remained unaltered compared to the unstimulated COX-2 null MEFs. Similarly, IL-1β stimulation significantly increased the levels of 6-keto PGF_{1α} in COX-2 WT MEFs. However, the levels of 6-keto PGF_{1α} were almost undetectable in IL-1β stimulated and unstimulated COX-2 null MEFs (Fig 5B).

Effect of mPGES-1 genetic deletion and subsequent decrease in PGE₂ production on iNOS and eNOS expression

eNOS and iNOS are the key NOS enzymes known to be involved in various physiological and pathophysiological processes. Recent studies have suggested crosstalk between NOS, PGE₂ and PG biosynthetic enzymes in inflammatory conditions (23). Therefore, we investigated the effect of mPGES-1 genetic deletion on the expression level of eNOS and iNOS enzymes.

eNOS was expressed constitutively in mPGES-1 MEFs, as no change in the eNOS expression was observed with mPGES-1 gene deletion or by IL-1β stimulation. iNOS protein expression was undetectable in unstimulated mPGES-1 WT and null MEFs (Fig. 6). However, on stimulation with IL-1β there was an induction of iNOS protein (12 h post IL-1β stimulation). iNOS protein expression levels were higher in the mPGES-1 null MEFs compared to their WT counterparts, indicating that mPGES-1 may in part be responsible for the differential expression of iNOS.

We further investigated the contribution of COX-2 (upstream enzyme to mPGES-1) toward the differential regulation of iNOS expression observed in mPGES-1 WT and null MEFs using nonselective COX and selective COX-2 inhibitors. Results showed that incubation of IL-1β-stimulated mPGES-1 MEFs with indomethacin and NS-398 increased the expression levels of iNOS in only mPGES-1 WT MEFs, elevating the levels similar to that observed in null MEFs (Fig. 6). These results demonstrate that in the absence of mPGES-1 deletion (as observed in mPGES-1 WT MEFs), inhibition of COX-2 contributes toward the up-regulation of iNOS expression. Similarly, in the presence of mPGES-1 genetic deletion (mPGES-1 null MEFs), nonselective COX and selective COX-2 inhibitors exhibit no effect on iNOS expression. These results indicate that mPGES-1 and COX-2-derived PGE₂ contribute to the regulation of iNOS in MEFs.

To further evaluate the contribution of PGE₂ toward regulation of iNOS, we determined the effects of exogenous PGE₂ on iNOS expression. PGE₂ treatment substantially decreased the level of iNOS expression in mPGES-1 null MEFs, bringing down the levels similar to WT MEFs, while no change in the levels of iNOS expression was observed in mPGES-1 WT MEFs. Thus, these results clearly suggest that mPGES-1 and COX-2-derived PGE₂ negatively regulate iNOS expression in MEFs.

Differential level of nitrite biosynthesis in mPGES-1 WT and null MEFs

NO generated by metabolism of L-arginine by NOS enzyme is highly unstable and is rapidly converted into stable breakdown products, nitrite, and nitrate. Therefore, in the present study we assessed the effect of mPGES-1 deletion on the levels of nitrite in mPGES-1 WT and null MEFs. Results showed that low levels of nitrite were detectable in unstimulated mPGES-1 WT and null MEFs. However, stimulation with IL-1 β resulted in a significant increase in the levels of nitrite in both mPGES-1 WT and null MEFs (Fig. 7). Nitrite levels in the mPGES-1 null MEFs were significantly higher compared to their WT counterparts, concordant with iNOS expression data.

Similarly, as observed with iNOS expression data, a substantial elevation in the levels of nitrite was observed in IL-1 β stimulated mPGES-1 WT MEFs treated with indomethacin and NS-398 (Fig. 7). However, indomethacin and NS-398 treatment showed no significant up-regulation in the levels of nitrite in IL-1 β stimulated mPGES-1 null MEFs, suggesting that there was little further contribution of COX inhibition when mPGES-1 was deleted. In addition, treatment with exogenous PGE₂ reduced the levels of nitrite in mPGES-1 null MEFs only, bringing the levels similar to mPGES-1 WT MEFs. These results further indicate that mPGES-1 and COX-2-derived PGE₂ negatively regulate iNOS expression and nitrite synthesis during inflammation.

DISCUSSION

This study using MEFs isolated from mPGES-1-deficient mice clearly draws out three major conclusions. First, genetic deletion of mPGES-1 results in a dramatic decrease in PGE₂ production in mPGES-1 Het and null MEFs in a gene dose-dependant manner, under basal and IL-1 β stimulation, suggesting that mPGES-1 is critical for PGE₂ production in these cells. Second, this study demonstrates a shunting phenomenon within the AA metabolic pathway as a result of mPGES-1 gene deletion with higher levels of 6-keto PGF_{1 α} in mPGES-1 Het and null MEFs compared to their WT counterparts, also in a gene dose-dependent manner. Third, we show that mPGES-1 gene deletion and resultant decrease in PGE₂ production results in a differential expression profile of iNOS and nitrite levels in mPGES-1 WT MEFs compared with null MEFs. Furthermore, using nonselective and selective COX-2 inhibitors and treatment with exogenous PGE₂, we show that COX-2- and mPGES-1-derived PGE₂ acts as a negative regulator of iNOS expression and nitrite synthesis during an inflammatory insult in mPGES-1 MEFs.

mPGES-1 is critical for PGE₂ production

We and others have previously shown that increase in PGE₂ production is associated with up-regulation of mPGES-1 along with COX-2 in variety of cells and tissues including synovial fibroblasts (14, 15, 21, 24) and synovial tissue (13, 25) isolated from patients with RA and OA, articular cartilage and chondrocytes isolated from patients with OA (20, 24). The present study for the first time demonstrates the role of mPGES-1 in fibroblast biology using mPGES-1 null MEFs. Results show that coordinated induction of COX-2 and mPGES-1 is concomitantly accompanied by the increase in PGE₂ levels in MEFs. Using cytokine-stimulated and unstimulated mPGES-1 Het and null MEFs, we show that PGE₂ levels significantly drop as a result of mPGES-1 gene deletion in a gene dose-dependent fashion, further emphasizing the fact that mPGES-1 along with COX-2 is critical for PGE₂ synthesis under basal as well as inflammatory conditions. This is despite the fact that COX-1, cPGES and mPGES-2 are expressed in these cells. These results support the studies by other groups that have also shown that mPGES-1 is essential for PGE₂ production and deletion of mPGES-1 gene results in a dramatic decline in PGE₂ levels in peritoneal macrophages (16, 17, 26–28) and microglia (29).

Shunting of endogenous prostanoid biosynthesis with mPGES-1 gene deletion

Using mPGES-1 WT, Het, and null MEFs in the present study, we show that genetic deletion of mPGES-1 results in the diversion of the prostanoid production profile from predominant PGE₂ to 6-keto PGF_{1α} under basal as well as proinflammatory cytokine-stimulated conditions in a gene dose-dependent manner without any significant changes in the levels of other prostanoids including PGD₂ and TXB₂. We also investigated if PGIS was up-regulated in mPGES-1 null MEFs resulting in the redirection of PGE₂ to 6-keto PGF_{1α} following mPGES-1 gene deletion. However, no significant changes in the mRNA and protein levels of PGIS and other PG synthetic enzymes (mPGES-2, cPGES, PGIS, h-PGDS, TXS, and COX-1) were observed with mPGES-1 gene deletion under basal or IL-1β stimulated conditions. These results indicate that in the absence of induced mPGES-1, elevation of COX-2 after inflammatory insult and the resultant increase in the availability of PGH₂ as the common substrate for the generation of other prostanoids account for increased levels of 6-keto PGF_{1α} observed with mPGES-1 deletion in mPGES-1 Het and null MEFs.

A previous study using mouse peritoneal macrophages derived from mPGES-1 null mice (DBA/11acJ genetic background) also supports the presence of shunting in the prostanoid biosynthesis (26). That study showed that PGE₂ was the predominant prostanoid produced in the lipopolysaccharide (LPS)-stimulated mPGES-1 WT mouse peritoneal macrophages. In contrast, incubation of mPGES-1 null mouse peritoneal macrophages with LPS resulted in a significant decrease in the levels of PGE₂ but significant increase in the levels of other prostanoids in the order of TXB₂ > 6-keto PGF_{1α} > PGF_{2α} > PGD₂ > PGE₂. This study also showed that the mPGES-1 gene deletion did not result in any change in the expression levels of PGIS and TXS.

Another study using mouse peritoneal macrophages isolated from mPGES-1 null mice (129P2×C57Bl/6 genetic background) also showed that PGE₂ was the predominant prostanoid produced in the LPS-stimulated mPGES-1 WT mouse peritoneal macrophages

(27). However, mPGES-1 gene deletion resulted in a significant increase in the levels of $\text{PGF}_{2\alpha}$, but only numerical increase in the levels of TXB_2 and no change in the levels of 6-keto $\text{PGF}_{1\alpha}$ in mPGES-1 Het and null mouse peritoneal macrophages compared to their WT counterparts. These two studies (26, 27) in comparison with the present study emphasize the cell specificity of prostanoid redirection.

mPGES-1- and COX-2-derived PGE_2 regulates iNOS expression and nitrite biosynthesis in mPGES-1 MEFs

iNOS is not normally expressed under basal conditions in tissues and cells but is highly inducible in response to inflammatory cytokines and bacterial endotoxins (30). iNOS catalyzes the conversion of L-arginine to L-citrulline and NO (31). NO is a highly unstable free radical gas that is rapidly converted into stable breakdown products called nitrite and nitrate. NO is involved in the physiological regulation of the vasculature (vasodilation), neurotransmission, host defense mechanisms, and various pathophysiological processes (32–34).

In this study for the first time, we provide evidence that mPGES-1 deletion and resultant decrease in PGE_2 lead to an up-regulation of iNOS expression and nitrite biosynthesis under proinflammatory cytokine-stimulated conditions. Data using NSAID (indomethacin and NS-398) and PGE_2 -treated MEFs and in combination with or without mPGES-1 gene deletion further demonstrate that mPGES-1- and COX-2-derived PGE_2 negatively regulates iNOS expression and nitrite biosynthesis. Because high levels of 6-keto $\text{PGF}_{1\alpha}$ correlated with the high levels of nitrite observed in IL-1 β -stimulated mPGES-1 null MEFs compared with WT MEFs, we investigated whether 6-keto $\text{PGF}_{1\alpha}$ had any contribution toward the regulation of nitrite synthesis in these cells. Blocking high levels of 6-keto $\text{PGF}_{1\alpha}$ in mPGES-1 null MEFs by indomethacin and NS-398 treatments had no effect on the high levels of nitrite which remained unaltered. These results suggest that PGI_2 and PGE_2 may not be equivalently involved in the regulation of nitrite synthesis.

Previous studies have also suggested that COX-2-derived prostanoids, mainly PGE_2 , may be involved in the crosstalk with iNOS and NO during inflammatory conditions, predominantly in macrophages (23, 35–7). It has been shown that treatment with PGE_2 decreases the levels of iNOS expression and nitrite biosynthesis in LPS-stimulated J774 macrophages (37). On the other hand, endogenous NO also has the ability to suppress the production of PGE_2 in LPS-treated RAW 264.7 murine macrophages (35).

Pharmacological significance of prostanoid diversion associated with mPGES-1 deletion

PGE_2 is a key proinflammatory mediator derived from COX-2 and mPGES-1, which has been shown to be elevated in various chronic inflammatory disorders, including RA, OA, atherosclerosis, Alzheimer's disease, and various forms of cancers (14, 15, 20, 21, 24, 38–40). COX-2 selective inhibitors exert their antiinflammatory pharmacological effects by inhibiting the excessive production of PGE_2 at the site of inflammation. However, recent clinical studies have suggested serious cardiovascular side effects associated with the use of COX-2 inhibitors (4–6). One of the hypotheses put forward to explain the cardiovascular

effects of COX-2 inhibition is the loss of antithrombotic PGI₂ derived from endothelial COX-2, which plays a key role in the regulation of thrombogenesis (41).

In the present study we demonstrate that genetic deletion of mPGES-1 results in transition in the production pattern from PGE₂ to 6-keto PGF_{1α}, whereas pharmacological inhibition and genetic deletion of COX-2 results in dramatic decrease in the levels of not only PGE₂ but also 6-keto PGF_{1α}. In addition, our results demonstrate that mPGES-1 gene deletion causes elevation of nitrite levels. 6-keto PGF_{1α} and nitrite are stable metabolites of PGI₂ and NO, which are involved in vasodilatation and maintenance of vascular homeostasis (32, 42, 43). Clinical studies have also indicated the beneficial effects of PGI₂ and NO for the management of circulatory disorders (44). In view of the above facts, mPGES-1 pharmacological inhibition may not be associated with cardiovascular side effects seen with inhibition of COX-2. The efficacy of such a therapeutic strategy remains unclear since increased levels of PGI₂ and NO could also exert proinflammatory effects as observed in the studies using PGI₂ receptor and iNOS deficient mice (45–47). In light of our present results, however, inhibition of mPGES-1 continues to be an attractive therapeutic target for PGE₂ inhibition in inflammatory conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

B

Figure 1. Effect of IL-1β on mRNA expression of COX and terminal PG synthase in mPGES-1 WT and null MEFs. *A*) Genotype of mPGES-1 WT, Het, and null mice. mRNA levels of mPGES-1 (28 cycles) and GAPDH (20 cycles) from mPGES-1 WT, Het, and null MEFs were determined by RT-PCR. *B*) mPGES-1 WT and null MEFs were harvested at 0, 1, 3, 6, 12, and 24 h post IL-1β stimulation, and mRNA expressions of mPGES-1 (28 cycles), COX-2 (25 cycles), mPGES-2 (25 cycles), cPGES (30 cycles), PGIS (30 cycles), H-PGDS (31 cycles), TXS (30 cycles), COX-1 (30 cycles), and GAPDH (20 cycles) were detected by RT-PCR. Representative data from three separate embryo lines are shown.

Figure 2.

Effect of IL-1 β on protein expression of COX and terminal PG synthase in mPGES-1 WT and null MEFs. mPGES-1 WT and null MEFs were harvested at 0, 6, 12, 24, and 48 h post IL-1 β stimulation and protein expressions of mPGES-1, COX-2, mPGES-2, cPGES, PGIS, H-PGDS, TXS, COX-1, and β -actin were determined by Western blotting. Representative data from three separate embryo lines are shown.

Figure 3.

Differential production pattern of PGE₂ vs. 6-keto PGF_{1α} in mPGES-1 null MEFs. A) Levels of PGE₂, 6-keto PGF_{1α}, PGD₂, and TXB₂ in supernatants of mPGES-1 WT and null MEFs were detected by ELISA at 0, 6, 12, and 24 h post IL-1β stimulation. Data are expressed as the mean ± SEM for 3–4 embryo lines. *WT control vs. WT IL-1β; #null control vs. null IL-1β; \$WT control vs. null control; £WT IL-1β vs. null IL-1β. Single and double characters indicate significance at $P < 0.05$ and $P < 0.01$, respectively. B) Levels of PGE₂ and 6-keto PGF_{1α} in supernatants of mPGES-1 WT, Het, and null MEFs were detected by

ELISA at 12 h post IL-1 β stimulation. Data are expressed as the mean $\pm$ SEM for three embryo lines. *, **Indicate statistical significance at $P < 0.05$ and $P < 0.01$, respectively.

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Figure 4.

Effect of indomethacin and NS-398 on PGE₂ and 6-keto PGF_{1α} production in mPGES-1 WT and null MEFs. mPGES-1 WT and null MEFs were incubated with or without IL-1β (1 ng/ml) for 12 h in the presence or absence of indomethacin (1 μM) or NS-398 (5 μM). Levels of PGE₂ and 6-keto PGF_{1α} in supernatants were detected by ELISA. Data are expressed as the mean ± SEM for three embryo lines. *, ** Indicate statistical significance at $P < 0.05$ and $P < 0.01$, respectively.

Figure 5.

Levels of PGE₂ and 6-keto PGF_{1α} production in COX-2 WT, Het, and null MEFs. A) Genotyping of COX-2 WT, Het, and null mice. Protein levels of COX-2 in COX-2 WT, Het, and null MEFs were determined by Western blotting. Ovine standard COX-2 protein was used as a positive control. B) COX-2 WT, Het, and null MEFs were incubated with IL-1β (1 ng/ml) for 12 h. Levels of PGE₂, 6-keto PGF_{1α} in supernatants were detected by ELISA. Data are expressed as the mean $\pm$ SEM for three embryo lines. *, ** Indicate statistical significance at $P < 0.05$ and $P < 0.01$, respectively.

Figure 6. Levels of iNOS and eNOS expression in mPGES-1 WT and null MEFs: Effect of NSAIDs and PGE₂ mPGES-1 WT and null MEFs were incubated with or without IL-1 β (1 ng/ml) for 12 h in the presence or absence of indomethacin (1 μ M), NS-398 (5 μ M), and PGE₂ (1 μ M) and protein expressions of iNOS, eNOS, and β -actin were determined by Western blotting. Representative blots from three embryo lines are shown.

Figure 7. Differential levels of nitrite in mPGES-1 WT and null MEFs: Effect of NSAIDs and PGE₂ mPGES-1 WT and null MEFs were incubated with or without IL-1β (1 ng/ml) for 12 h in the presence or absence of indomethacin (1 μM),NS-398 (5 μM), and PGE₂ (1 μM). Levels of nitrite in supernatants were detected by ELISA. Data are expressed as the mean ± SEM for three embryo lines. *, ** Indicate statistical significance at *P* < 0.05 and *P* < 0.01, respectively.

Schematic diagram.

Prostanoid diversion profile in mPGES-1 deletion versus COX-2 deletion/inhibition: regulation of iNOS and NO.