Deletion of Microsomal Prostaglandin E Synthase-1 Does Not Alter Ozone-Induced Airway Hyper-Responsiveness^[S]

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ABSTRACT

Nonsteroidal anti-inflammatory drugs ameliorate pain and fever by inhibiting cyclooxygenase (COX) and suppressing prostanoid formation. Microsomal prostaglandin E synthase-1 (mPGES-1) catalyzes formation of PGE₂ from the COX product PGH₂ and has emerged as a therapeutic target. Inhibition of mPGES-1, however, renders the PGH₂ substrate available for diversion to other PG synthases. To address the possibility that substrate diversion augments formation of PGs that might modulate bronchial tone, we assessed the impact of mPGES-1 deletion in a mouse model of ozone-induced airway hyper-responsiveness. Ozone exposure increased total lung resistance to inhaled methacholine in wild-type mice. Deletion of mPGES-1 had little effect on total lung resistance in either naive or ozone-exposed animals.

The carbachol-induced narrowing of luminal diameter in intrapulmonary airways of lung slices from acute ozone-exposed mice was also unaltered by mPGES-1 deletion. Likewise, although concentrations of PGE₂ were reduced in bronchoalveolar lavage fluid, whereas 6-keto-PGF_{1α}, PGD₂, and PGF_{2α}, all were increased, deletion of mPGES-1 failed to influence cell trafficking into the airways of either naive or ozone-exposed animals. Despite biochemical evidence of PGH₂ substrate diversion to potential bronchomodulator PGs, deletion of mPGES-1 had little effect on ozone-induced airway inflammation or airway hyper-responsiveness. Pharmacologically targeting mPGES-1 may not predispose patients at risk to airway dysfunction.

Prostaglandins (PGs) are a group of bioactive lipids formed by the sequential enzymatic actions of cyclooxygenases (COXs) and terminal synthases that convert the COX product PGH₂ to specific PGs. Placebo-controlled trials have revealed that nonsteroidal anti-inflammatory drugs (NSAIDs) selective for inhibition of COX-2 confer a cardiovascular hazard (FitzGerald, 2007). Integration of

diverse lines of evidence (Grosser et al., 2010) indicates that this is consequent to suppression of cardioprotective PGs, particularly prostacyclin (PGI₂). Microsomal PGE synthase-1 (mPGES-1) (Jakobsson et al., 1999; Thorén et al., 2003), an enzyme downstream in the biosynthetic cascade, catalyzes the isomerization of PGH₂ into PGE₂ and is a member of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) superfamily. It has been suggested to be an anti-inflammatory drug target alternative to NSAIDs (Jakobsson et al., 1999; Samuelsson et al., 2007). Two other PGE synthases have been identified, mPGES-2 (Murakami and Kudo, 2006) and cytosolic PGES (Tanioka et al., 2000; Pini et al., 2005). mPGES-1, however, is the dominant source of PGE₂ biosynthesis, at least in mice (Cheng et al., 2006). Although some have found that mPGES-1 deletion modulates experimentally evoked pain and arthritis to a degree indistinguishable from treatment with traditional NSAIDs (tNSAIDs)

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ABBREVIATIONS: COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; tNSAID, traditional NSAID; PG, prostaglandin; mPGES-1, microsomal PGE synthase-1; PGI₂, prostacyclin; BAL, bronchoalveolar lavage; PCLS, precision-cut lung slices; WT, wild type; KO, knockout; ANOVA, analysis of variance; MO, methoxime; FA, forced air; Tx, thromboxane.

(Trebino et al., 2003; Kamei et al., 2004) and restrains immune-induced pyresis (Engblom et al., 2003), others have questioned the analgesic efficacy of this approach (Scholich and Geisslinger, 2006). However, in contrast to the effects of inhibition or deletion of COX-2, deletion of mPGES-1 does not enhance responsiveness to a thrombogenic stimulus in vivo (Cheng et al., 2006). Furthermore, deletion of mPGES-1 retards atherogenesis in hyperlipidemic mice (Wang et al., 2006) and ameliorates experimentally induced abdominal aortic aneurysm (Wang et al., 2008). These cardiovascular properties may reflect substrate diversion, augmenting formation of cardioprotective PGs, particularly PGI₂ (Cheng et al., 2006; Wang et al., 2008). Given that those studies suggest the functional importance of substrate diversion when mPGES-1 is deleted or inhibited, we wanted to address the possibility that the same mechanism might also confer risk. Thus, augmented formation of potential bronchoconstrictor prostanoids, such as PGD₂, (Oguma et al., 2008), might predispose patients at risk to airway hyper-responsiveness. Deletion of the DP1 receptor for PGD₂ in mice sharply reduces allergen-induced infiltration of lymphocytes and eosinophils and airway hyper-reactivity (Matsuoka et al., 2000). It is unknown whether targeting mPGES-1 may affect airway function in a high-risk condition. To address this possibility, we assessed the impact of mPGES-1 deletion on airway hyper-reactivity induced by ozone. Ozone exposure increases airway responsiveness to inhaled methacholine in humans (Seltzer et al., 1986; Coffey et al., 1996) and animals (Holroyde and Norris, 1988) and is associated with an influx of neutrophils into the airway. In this article, we report that, despite augmented formation of PGD₂, mPGES-1 deletion in mice had little effect on ozone-induced hyper-responsiveness and airway inflammation.

Materials and Methods

Mice and Ozone Treatment. mPGES-1-deficient mice [mPGES-1(-/-)] and their controls [mPGES-1(+/-)] were generated by corresponding homozygous breeders that were derived from intercrossing mPGES-1(+/-) mice, which are derived from the original mPGES-1(-/-) mice on a DBA background (Trebino et al., 2003) and backcrossed to C57BL/6 for seven generations. Female mice were exposed for 2 h to ozone at 6 ppm or forced air (FA) while being deprived of food and water. Ozone was generated as described previously (Kierstein et al., 2006, 2008). This concentration of ozone induces an increase in murine airway sensitivity to a contractile agonist without the presence of immediate infiltrating inflammatory cells, as previously determined in an assessment of precision-cut lung slices (PCLS) airway function (Cooper et al., 2010). Ozone treatment also induces delayed airway inflammation and hypersensitivity, and this was studied in mice 18 h after the exposure by measuring respiratory mechanics.

Mice were euthanized after assessment of lung function, and bronchoalveolar lavage (BAL) fluid was collected by perfusing the lung three times with two 1.0-ml aliquots of phosphate-buffered saline through a syringe attached to the trachea. Recovered BAL fluid was centrifuged, and the supernatant was analyzed for eicosanoids, cytokines, and/or protein content. The cell pellet was resuspended and processed for total and differential cell counts by routine methods. All animals were housed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania, and all experiments were approved by the committee.

Measurements of Airway Resistance and Compliance in Anesthetized Mice. Direct measurements of respiratory system

mechanics in response to acetyl- β -methylcholine chloride were made by using the FlexiVent system (SCIREQ, Montréal, QC, Canada). FlexiVent uses a low-frequency forced-oscillation technique to measure respiratory system input impedance and evaluate the constant-phase model. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline. A tracheostomy was performed by inserting a 20-gauge polyethylene catheter into the distal trachea and ligating it around the catheter to avoid any leaks or a disconnection from the ventilator. The animals were then mechanically ventilated with a computer-controlled small-animal ventilator (SCIREQ) with a rate of 150 breaths/min, tidal volume of 10 ml/kg, and peak expiratory pressure of 2 to 3 cm of H₂O. Once ventilated, mice were paralyzed with 0.8 mg/kg pancuronium bromide to block spontaneous breathing. Using the custom-designed software FlexiVent 5.2 (SCIREQ), e.g., SnapShot-150, resistance, elastance, and compliance were recorded, and input impedance was measured with Quick Prime-3, to distinguish between central airway and lung tissues. Before starting each dose measurement, total lung capacity was performed twice to open up the airspace and standardize lung volume for baseline determination. Then, aerosolized methacholine at doses of 0, 2.5, 5, 10, 20, 50, and 100 mg/ml was delivered via an in-line nebulizer in the ventilator circuit for 10 s. After methacholine exposure, 12 measurements were taken over 3 min for each dose.

PCLS Preparation and Intrapulmonary Airway Function Assessment. In a different group of mice PCLS were prepared immediately after ozone exposure (2 h, at 6 ppm), with slight modifications, as described previously (Ressmeyer et al., 2006; Cooper et al., 2010). In brief, the trachea was exposed and intubated with a cannula, and the lungs were inflated with 0.65 ml of 2% (w/v) low melting point agarose solution (37°C) followed by 0.1-liter bolus of air to force the agarose out of the airways and into the parenchymal tissue. After allowing the agarose to set at 4°C, the lobes were separated, and the largest lobe was embedded externally in agarose by using a tissue-embedding unit (TSE Systems, Chesterfield, MO). PCLS (thickness 250 μ m) were prepared with a Krumdieck tissue slicer (model MD4000; Alabama Research and Development, Munford, AL) with the speed set to produce slices at approximately one every 30 s. Slices were transferred in sequence to wells containing supplemented Ham's F-12 medium and then incubated at 37°C on a rotating platform in a humidified air/CO₂ (95:5%) incubator. Media were changed every hour for 4 h to minimize trauma, reduce airway tone, and remove any remaining agarose in the tissue. Media were also changed the next day. Up to four slices from each animal were placed in a 12-well plate in 1.0 ml of buffer and held in place by using a platinum weight with nylon attachments. Airway function was measured as described previously (Cooper and Panettieri, 2008; Cooper et al., 2009) by using a microscope (ECLIPSE, model TE2000-U; Nikon, Melville, NY) connected to a live video feed (RETIGA-2000R video recorder; QImaging, BC, Canada). Images were collected 4 min after each dose of carbachol or until no further contraction was evident. Log EC₅₀ and E_{max} values for each airway were derived from a concentration-response curve, and mean values for each animal were also derived.

Measurement of Eicosanoids and Proteins. BAL levels of PGE₂, 6-keto-PGF_{1 α} (the stable inactive hydrolysis product of PGI₂), PGD₂, PGF_{2 α} , and thromboxane (Tx) B₂ (the hydrolysis product of TxA₂) were quantified by ultra high-pressure liquid chromatography/tandem mass spectrometry, using solid-phase extraction, negative ion electrospray introduction, and selected reaction monitoring techniques. Tetradeuterated analogs of PGD₂, PGE₂, PGF_{2 α} , 6-keto-PGF_{1 α} , and TxB₂ (Cayman Chemical, Ann Arbor, MI), 5 ng each, were added to 0.6 ml of BAL. The methoxime (MO) derivative was formed by adding 0.3 ml of methoxyamine HCl (1g/ml) in water, and eicosanoids were extracted on StrataX solid-phase extraction cartridges (Phenomenex, Torrance, CA), then dissolved in 200 μ l of 20%

acetonitrile in water for analysis. The instrument used was a Quantum Ultra interfaced to an Accela ultra high-pressure liquid chromatography system (Thermo Fisher Scientific, Waltham, MA). A

200-mm $\times$ 2.1-mm $\times$ 1.9- μ m Hypersil Gold column (Thermo Fisher Scientific) was used. The mobile phase was generated from high-performance liquid chromatography-grade water (A) and 5% meth-

Fig. 1. Levels of prostanooids in BAL fluid. BAL samples were isolated from FA or ozone-exposed mPGES-1 WT [mPGES-1(+/+)] and KO [mPGES-1(-/-)] mice ($n = 10$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. These labels apply to all figures.

Fig. 2. Lung resistance (a) and dynamic compliance (b) measured after exposure of mice to methacholine. Response to each methacholine dose was plotted as the mean $\pm$ S.D. for each group ($n = 5$). FA: control for ozone exposure; BL: baseline. One outlier at a dose of 50 mg/ml that gave an enormously high value to the group of WT plus ozone was excluded. There is no statistical significance between WT and KO at any given dose of methacholine in control or ozone exposure condition.

anol/95% acetonitrile (B), both containing 0.005% acetic acid adjusted to pH 5.7 with ammonium hydroxide. The flow rate was 350 μ l/min using a segmented linear gradient starting at 20% (T = 0), ramping to 35% B (T = 15 min), 40% B (T = 16 min) then 70% B (T = 23 min). The transitions monitored were m/z 384 $\rightarrow$ 272 (d_4 -PGD₂ MO and d_4 -PGE₂ MO), m/z 380 $\rightarrow$ 268 (PGD₂ MO and PGE₂ MO), m/z 357 $\rightarrow$ 197 (d_4 -PGF_{2 α}), m/z 353 $\rightarrow$ 193 (PGF_{2 α}), m/z 402 $\rightarrow$ 173 (d_4 -TxB₂ MO), m/z 398 $\rightarrow$ 169 (TxB₂ MO), m/z 402 $\rightarrow$ 372 (d_4 -6-keto-PGF_{1 α} MO), and 398 $\rightarrow$ 368 (6-keto-PGF_{1 α} MO). The collision gas was argon, 1.5 mTorr. The collision energy was 18 eV for PGD₂, PGE₂, 6-keto-PGF_{1 α} , and TxB₂, and 24 eV for PGF_{2 α} . Source offset was 6 V. Quantitation was by peak area ratios. Levels of mouse CXC chemokine KC and total protein in BAL fluid were determined with an enzyme-linked immunosorbent assay kit (R&D Systems, Minne-

apolis, MN) and Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA), respectively.

Statistical Analysis. Data are expressed as means $\pm$ S.E.M. unless indicated otherwise. Statistical comparisons among treatment groups in reactivity to methacholine were performed by two-way ANOVA, followed by the Newman-Keuls post hoc test for more than two groups. Comparisons of multiple groups were performed by one-way ANOVA and Bonferroni post-ANOVA test when the ANOVA was deemed significant. When only two mean values were compared, the two-tailed Mann-Whitney t test was used. In all cases, statistical significance was defined as $p < 0.05$.

Results

Deletion of mPGES-1-Modulated Airway Production of Prostanoids. Evidence for substrate diversion consequent to mPGES-1 deletion was sought in BAL (Fig. 1). In both FA and ozone-exposed animals, deletion of mPGES-1 significantly reduced PGE₂, while augmenting biosynthesis of PGI₂, PGD₂, and PGF_{2 α} .

Deletion of mPGES-1 Had Little Effect on Modulating Ozone-Induced Airway Responsiveness. To study whether targeting mPGES-1 may affect airway function in a predisposed high-risk condition, ozone-induced airway hyper-responsiveness was evaluated in mice deficient in mPGES-1 (Fig. 2). Enzyme deletion did not affect total lung resistance after challenge with increasing doses of methacholine in either naive or ozone-exposed animals, despite a hyper-responsiveness induced by ozone exposure (Fig. 2a). Likewise, mPGES-1 deletion failed to modulate significant compliance in naive or ozone-exposed animals (Fig. 2b).

Although ozone treatment increased carbachol-induced

Fig. 3. EC₅₀ of intrapulmonary airway contraction to carbachol. Intrapulmonary airways were prepared from WT and KO mice after exposure to ozone or FA for 2 h at 6 ppm ($n = 5$ mice per group). * and ** denote statistical significance compared with the corresponding genetic phenotype, i.e., WT versus WT and KO versus KO. There is no statistical significance between ozone WT versus ozone KO.

Fig. 4. mPGES-1 in ozone-induced airway inflammation. Total inflammatory cell count in BAL fluid (a), differentiated BAL cell populations (b), and their percentage of total BAL cell (c) are shown.

contraction of intrapulmonary airways in sliced lung sections, as reflected by a significant decrease in EC_{50} (log EC_{50} from 0.04 ± 0.11 to $-0.36 \pm 0.12 \mu M$), mPGES-1 deletion had little effect on the sensitivity to carbachol-induced intrapulmonary airway contraction (Fig. 3).

Deletion of mPGES-1 Did Not Affect Ozone-Induced Airway Inflammation. Ozone exposure increased inflammatory cell trafficking into the BAL (by ~ 6 fold) as shown in Fig. 4a. The inflammatory cell infiltrate was composed primarily of neutrophils ($\sim 55\%$) and macrophages ($\sim 43\%$) (Fig. 4b). However, no differences in total BAL cell count or the differential macrophage, eosinophil, neutrophil, or lymphocyte counts were observed between wild-type (WT) and knockout (KO) animals (Fig. 4). Consistently, levels of KC (a neutrophil chemoattractant) and total protein in BAL were not changed in the KO animals (Supplemental Figure).

Discussion

Selective inhibitors of COX-2 were developed with the aim of conserving efficacy but reducing the incidence of serious gastrointestinal adverse events consequent to use of tNSAIDs, such as ibuprofen, which inhibited coincidentally COX-1 and COX-2. Although two of these newer drugs, rofecoxib and lumiracoxib, have been shown in randomized trials to cause serious gastrointestinal complications less frequently than tNSAID comparators, evidence has emerged that selective inhibition of COX-2 by the newer coxibs and some tNSAIDs may result in a cardiovascular hazard attributable to suppression of PGI_2 (Grosser et al., 2010). mPGES-1 deletion, by contrast, augments PGI_2 formation caused by diversion of the PGH_2 substrate to PGI isomerase. Ironically, this tends to undermine the original intent of developing mPGES-1 inhibitors as PGI_2 , such as PGE_2 , that can mediate pain and inflammation (Murata et al., 1997; Honda et al., 2006). However, evidence in mice suggests that augmentation of PGI_2 synthesis may not just attenuate the thrombogenic and hypertensive hazard seen with COX-2 inhibition, but actually restrains atherogenesis and aneurysm formation, suggesting that mPGES-1 inhibitors may have a role in altering cardiovascular inflammation.

Although the emphasis has been on substrate diversion to PGI_2 , we also found (Wang et al., 2008) that biosynthesis of PGD_2 was augmented by mPGES-1 deletion. Although activation of the adenylate cyclase-coupled platelet DP1 receptor for this PG may contribute to cardioprotection, this finding also raised the possibility of pulmonary adverse effects of mPGES-1 inhibitors. Others have shown that augmented PGD_2 can provoke airway constriction, and $PGF_{2\alpha}$, another potential product of substrate redirection, also causes bronchoconstriction in vitro (Mathé et al., 1973). Here, we confirmed that deletion of mPGES-1 did indeed cause substrate diversion in the lung, as reflected by measurement of eicosanoids in BAL. We also found that residual PGE_2 (approximately 50% of the total PGE_2) was present in the mPGES-1 KO mice. PGE_2 can be also formed by mPGES-2 and cytosolic PGE synthase. These would be unaffected by the gene deletion or the specific mPGES-1 inhibitors under development. Despite the presence of these enzymes, however, deletion of mPGES-1 and/or its inhibition resulted in analgesic and anti-inflammatory effects in a variety of models (Xu et al., 2008; Mbalaviele et

al., 2010). These studies indicate that the reduction of PGE_2 levels in the KO mice is biologically significant.

Despite increased formation of both PGD_2 and $PGF_{2\alpha}$ in the mPGES-1 KO mice, airway function at baseline or in response to provocation with ozone was unaltered by mPGES-1 deletion. Our results may reflect a balanced increase in the bronchoconstrictor prostanoids and the bronchodilator PGI_2 . Indeed, recently, it has also been reported that PGI_2 inhibits allergen-induced airway inflammation (Jaffar et al., 2002; Takahashi et al., 2002; Nagao et al., 2003; Idzko et al., 2007), and despite the impact of DP1 deletion on allergen-induced airway hyper-responsiveness (Matsuoka et al., 2000), an anti-inflammatory function of PGD_2 , acting on its DP1 receptor on bone marrow-derived cells, has been described previously (Hammad et al., 2007). Furthermore, although PGE_2 might act as a bronchodilator via its EP2 and EP4 receptors, it may also have contributed to bronchoconstrictor tone via the EP1 and EP3 receptors (Narumiya et al., 1999; Tilley et al., 2003). It is likely that PGE_2 does not act as a direct dilator but as an endogenous "brake" on bronchoconstrictive agonists (Sestini et al., 1996). Prostanoids differentially generated along the COX-1 and COX-2 pathways may have distinct roles in airway inflammation and hyper-responsiveness (Swedin et al., 2009). BAL fluid is an indirect reflection of prostanoid generation in the lung and may not reflect regional disparities in product formation relevant to airway responsiveness. However, the altered impact on both constrictor and dilator prostanoids reflected in BAL may also explain the failure of mPGES-1 deletion to alter airway function.

Ozone-induced airway hyper-responsiveness in mice is but one model of evoked bronchoconstriction. However, at least in the present studies, despite substrate diversion to other prostanoids, suppression of PGE_2 by deleting mPGES-1 provided no evidence suggestive of enhanced airway dysfunction.

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